Measures of osteoarthritis in the horse

Volume One of One

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Abstract

In equine medicine there is a need to identify osteoarthritis at an early stage in order to improve the effectiveness of therapy. Therapies require accurate assessment necessitating well run clinical trials. For both these purposes it is necessary to use valid and reliable measures of the disease process. A pilot clinical study was conducted which allowed the requirements for a good clinical trial to be assessed, and evaluated the use of lameness scoring, owner questionnaires, radiography, scintigraphy and biochemical markers in the measurement of the disease, while investigating the effect of calcium pentosan polysulphate in cases of osteoarthritis in horses. In order to establish good guidelines for trial protocols the hypothesis that "joints are biologically different" was explored.

The assessment of functional outcome by lameness scoring and questionnaires, and the use of a radiographic scoring system were found to be reliable measurement tools. Scintigraphy was helpful diagnostically but requires further investigation to be useful in assessment of disease change. Keratan sulphate (5D4) and bone specific alkaline phosphatase were found to correlate well with degree of articular cartilage damage. Cross sectional differences between osteoarthritic and contralateral joints were found in synovial fluid levels of hyaluronan, keratan sulphate, total glycosaminoglycans and bone specific alkaline phosphatase, but these markers were not useful in assessing temporal changes during the trial. Marked differences were identified in concentration of the above synovial fluid markers between normal equine metacarpophalangeal, proximal interphalangeal, and distal interphalangeal joints, and normal ranges were established . Articular cartilage from these same joints was found to respond differently when challenged by interleukin - 1 *in vitro*.

The results of this study indicate that equine clinical trials should be joint specific, and verify that lameness scoring is a valid measure of functional change, and radiography scoring is a reliable measure of structural outcome.

Dedication and acknowledgements

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Author's declaration

Apart from the help and advice acknowledged, this thesis represents the unaided work of the author and was not conducted in collaboration with any other person.

Catherine Jane Fuller BVSc MRCVS July 1998

The views expressed in this thesis are those of the author and not those of Bristol University.

Abbreviations used in this thesis

ABC	Antebrachiocarpal joint
AI	Activity index
AIMS	Arthritis impact measurement scale
AP	Alkaline phosphatase
BAP	Bone specific alkaline phosphatase
BSP	Bone sialoprotein
CaPPS	Calcium pentosan polysulphate
CI	Confidence interval
CMC	Carpometacarpal joint
COMP	Cartilage oligomeric matrix protein
CS	Chondroitin sulphate
CSD	Corticosteroid
DIP	Distal interphalangeal joint
DMOAD	Disease modifying osteoarthritis drug
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
FP	Femoropatellar joint
GAG	Glycosaminoglycans
HA	Hyaluronan
HAQ	Health assessment questionnaire
HAS	Serum hyaluronan
HASF	Synovial fluid hyaluronan
ID	Irish draught
IGD	Interglobular domain
IL-1	Interleukin -1
K&L	Kellgren and Lawrence
KS	Keratan sulphate
kg	Kilogram
L	Litre
MC	Midcarpal joint
MCP	Metacarpophalangeal joint
MDP	Methylene diphosphonate
mg	Milligram
ml	Millilitre
MMP	Metalloproteinase
MTP	Metacarpal joint
NSAID	Non-steroidal anti-inflammatory drug

OA	Osteoarthritis
PG	Proteoglycan
PIP	Proximal interphalangeal joint
PPS	Pentosan polysulphate
PSGAG	Polysulphated glycosaminoglycan
RCT	Randomised controlled trial
SADOA	Slow acting drug in osteoarthritis
SD	Standard deviation
SEM	Standard error of the mean
SF	Synovial fluid
SHA	Sodium hyaluronate
SIQR	Semi interquartile range
SRM	Standard response mean
SYSADOA	Symptomatic slow acting drug in osteoarthritis
TB	Thoroughbred
TC	Tarsocrural joint
Tc	Technetium
VAS	Visual analogue scale
WOMAC	Western Ontario and McMasters Universities health assessment questionnaire

Material already published from work contained in this thesis

From Chapter Seven of this thesis:

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Chapter One

Introduction

Where there is much desire to learn, there of necessity will be much arguing, much writing, many opinions; for opinion in good men is but knowledge in the making.

John Milton 1608 - 1674

Osteoarthritis - a definition?

The search for a generally accepted definition of osteoarthritis (OA) has occupied rheumatologists for many years.

In horses the condition that we now consider to be OA, was originally believed to be the sequel to acute rheumatism, a systemic "influenza-like" disease, although by the late 19th century some scientists were recognising it to be an "independent and idiopathic" disease (Robertson 1890). It was generally described as separate conditions according to joint site, with differing aetiological factors. In 1830 James White, veterinary surgeon with the Royal Dragoons, described bone spavin (OA of the tarsal joints) as "a very common disease, consisting of a deposit of bony matter about the bones of the hock" (tarsus) (White 1830). He proposed that spavin was caused by the obliteration of the synovial fluid producing gland situated in the distal tibia, following such long exertion that the synovia was consumed and the gland was unable to supply more. This resulted in inflammation, ulceration and ossification of the joints. In 1893 Mayhew describes spavin and ringbone (OA of the interphalangeal joints) as work related, man induced diseases. He suggested that spavin, common in carriage horses, was caused by exertion changing ligament into bone, and that ringbone, common in carthorses, was caused by force on the pastern bones causing inflammation and lymphatic effusion which changed into cartilage and then bone (Mayhew 1893). Smith (1893) classified ringbone and spavin as specific joint diseases in which "there is something in common in the nature of lesions produced which would lead one to suspect that the changes occurring in each are closely allied". It was originally thought that OA was a disease which commenced in the synovium or bone and rarely in the cartilage, since that was an avascular tissue and therefore least susceptible (Wortley 1910). In 1927, Hare carried out a post-mortem survey of 34 horses (Hare 1927) and reported that the original abnormality in "chronic rheumatism" was focal circumscribed areas of hyperaemic bone with corresponding focal areas of articular

cartilage fibrillation. He concluded " It is far easier to define what this arthritis is not, than to provide a positive definition which would find universal acceptance." His definition of OA was "a disease of joints tending to chronicity, which is probably specific but due to no known specific cause". It seems that little has changed in this respect over 100 years.

A recently proposed definition of OA in man, describes a disease process involving a disturbance in the normal balance of degradation and repair in the articular cartilage and subchondral bone (Goldberg *et al.* 1995). It has a different balance of risk factors and outcome at different joint sites and is characterised by the focal loss of cartilage with subchondral bone sclerosis, and marginal osteophyte formation (Dieppe 1991). All these processes are linked, although it is not known which are primary and which are secondary.

The disease has been described quite differently in human and equine OA but it is commonly agreed that it involves a loss in function of the articular cartilage. The relative age of equine patients is younger and the expectations of treatment are greater. Synovitis may be present in equine OA (McIlwraith 1996) whereas the disease is often cited as noninflammatory in human OA.

Impact on population

Osteoarthritis is one of the main causes of lameness in horses in the UK, causing 28% loss of training days in the Thoroughbred racehorse (Rossdale *et al.* 1985). In man the prevalence of radiographically defined OA ranges from 14 - 30% in men and women over 45 years (Hart *et al.* 1995) but prevalence is difficult to measure because of differences in the definition of OA. Definition should be by a combination of clinical and radiographical means, since clinical symptoms do not correlate well with radiographic changes, and in most elderly patients OA, as defined radiographically, may be present but is often asymptomatic.

Despite OA being the most common rheumatic disease, little is still known concerning its aetiology, natural history, and progression. Before well designed epidemiological studies can be carried out in order to promote greater understanding of the disease, a consistent well defined definition of the disease and standardised criteria for describing cases and measuring changes are necessary.

Joints are most commonly classified according to their degree of movement i.e.

1) synarthroses - immovable joints

2) amphiarthroses - slightly moveable joints

3) diarthroses - movable joints

Most of the joints in the extremities are diarthroses and it is these that will be referred to throughout this thesis. The joint is an organ whose purpose is to allow frictionless movement of the limbs while maintaining stability to the body. It is the unique properties of the tissues within this organ that combine to allow locomotion.

OA is a disease process characterised by a mixture of degradative and reparative processes in each of the three main elements of the synovial joint - cartilage, bone , and the synovial/capsular lining (Dieppe 1995) . The normal anatomy and function of these tissues will be reviewed here.

The diarthrodial (or synovial) joint

The diarthrodial joints differ from the synarthroses and amphiarthroses in that they consist of at least two articulating surfaces of bone which are covered in articular cartilage and separated by synovial fluid within a cavity surrounded by a joint capsule.

1. Cartilage

Hyaline articular cartilage has a structure which is highly suited to provide distribution of load and resistance to compressive forces, smooth articulation and cushioning of the subchondral bone (Kuettner *et al.* 1991). It does not contain blood vessels, nerves or lymphatics, and appears grossly and histologically to be a simple inert tissue (Buckwalter *et al.* 1997). It consists of one type of cell - the chondrocyte - which produces an extracellular matrix made up of a meshwork of collagen fibres and proteoglycan molecules. It has a low level of metabolic activity and is less responsive to injury than bone or muscle. It varies in thickness, cell density, matrix composition and mechanical properties within joints, between joints and between species but the general structure remains the same (Buckwalter and Mankin 1997). The biomechanical properties of cartilage depend on the composition of the extracellular matrix, a factor which is ultimately under the control of the chondrocyte.

Composition:

Cartilage consists of approximately 60 - 80% water, with the dry weight being composed of approximately 60% collagen, 25-35% proteoglycans, and 5-15% noncollageneous proteins. Histologically it is organised in four zones (see later) in each of which the exact

organisation of the cartilage constituents as well as its metabolic activity differs (Wong *et al.* 1996).

Chondrocytes

The chondrocyte is the only type of cell in the cartilage and individually each cell is highly metabolically active. However, the cell density within the cartilage is very low with the cells occupying only 2 -10 % of the tissue volume. The chondrocyte is responsible for the synthesis and constant replacement of all the matrix macromolecules, and must therefore sense changes in the molecular environment. Chondrocytes receive their nutrition from the synovial fluid, and these nutrients must first diffuse through the synovial tissue and fluid and then across the cartilage matrix. For this reason chondrocytes function at a low concentration of oxygen, depending primarily on anaerobic metabolism. The enzymes responsible for the degradation of matrix macromolecules are also produced by the chondrocytes, and the presence of fragments of these degraded molecules stimulate the cells to increase synthetic activity. However the capacity for synthesis of some molecules is not adequately maintained as the chondrocytes age and this factor may contribute to degeneration of the cartilage with age. Chondrocytes are heterogeneous cells with those in different cartilage zones synthesising different amounts of molecules and acting quite differently to the same stimuli.

Extracellular matrix

The matrix surrounding the chondrocytes consists of two very different but vitally interactive components - the tissue fluid and the framework of structural macromolecules.

Tissue fluid

The interaction of the tissue fluid with the macromolecular framework gives cartilage its mechanical properties of stiffness and resilience. The amount of water in the cartilage ranges from 60 - 80% of wet weight, and the exact amount depends largely on the interactions between electrolytes in the fluid and the large aggregating proteoglycans within the framework (Mow *et al.* 1992).

Structural macromolecules

a) Collagen

Collagens contribute about 60% of the dry weight of the cartilage and are distributed relatively uniformly throughout the tissue. The collagen fibrillar network is responsible for the form and tensile strength of the cartilage. The principal collagen in hyaline cartilage is Type II which accounts for 90% of the total collagen, although types VI, IX, X, and XI, also occur. The collagen is organised into a tight meshwork, which provides

tensile strength as well as trapping large proteoglycans within its structure. The strength of the meshwork is enhanced by covalent crosslinks between the collagen molecules.
The type II collagen molecule is composed of three identical α polypeptide chains wound in a left handed helix. Three α-chains are further arranged in a right handed triple helix to form a tropocollagen subunit (Mow *et al.* 1992). Collagen fibrils consist of a staggered array of these tropocollagen units (Figure 1- 1).

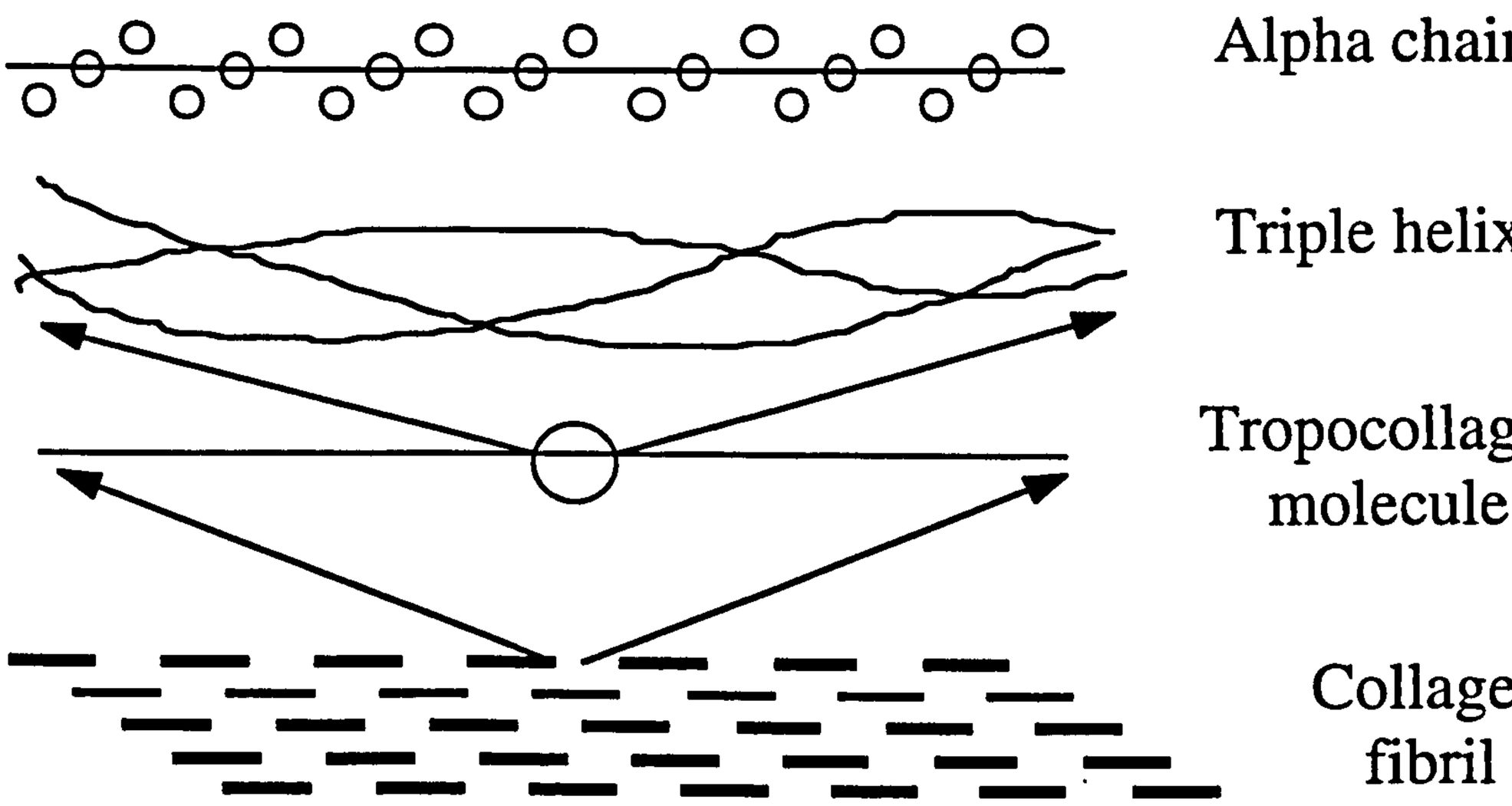

Figure 1-1: Structure of collagen fibril[1]

[1] Adapted from Collagen:Biochemistry,Vols I, II, and III, Nimni ME (ed), CRC Press, Boca Raton,FL, USA,1988

b) Proteoglycans

Proteoglycans contribute about 25 - 35% of the cartilage dry weight. They consist of a protein core and one or more glycosaminoglycan side chains. The glycosaminoglycans are composed of repeating disaccharides which have negatively charged sulphate or carboxyl groups and these highly charged molecules attract water and exert a swelling pressure. Proteoglycans are thus responsible for the compressive stiffness of hyaline cartilage. Large aggregating proteoglycans or "aggrecan" fill most of the interfibrillar space, accounting for about 90% of the total cartilage matrix proteoglycan mass. Most bind covalently with hyaluronan and are stabilised by link proteins (small non collagenous proteins). Small non aggregating proteins also exist, e.g. decorin, fibromodulin and biglycan. These have a shorter protein core than aggrecan and are thought to influence cell function rather than having any effect on the mechanical nature of cartilage. There are three major types of glycosaminoglycans in cartilage - chondroitin-4-sulphate, chondroitin-6-sulphate, and keratan sulphate. The proportion of these different glycosaminoglycan chains vary with zone of the cartilage as well as with age, injury and disease.

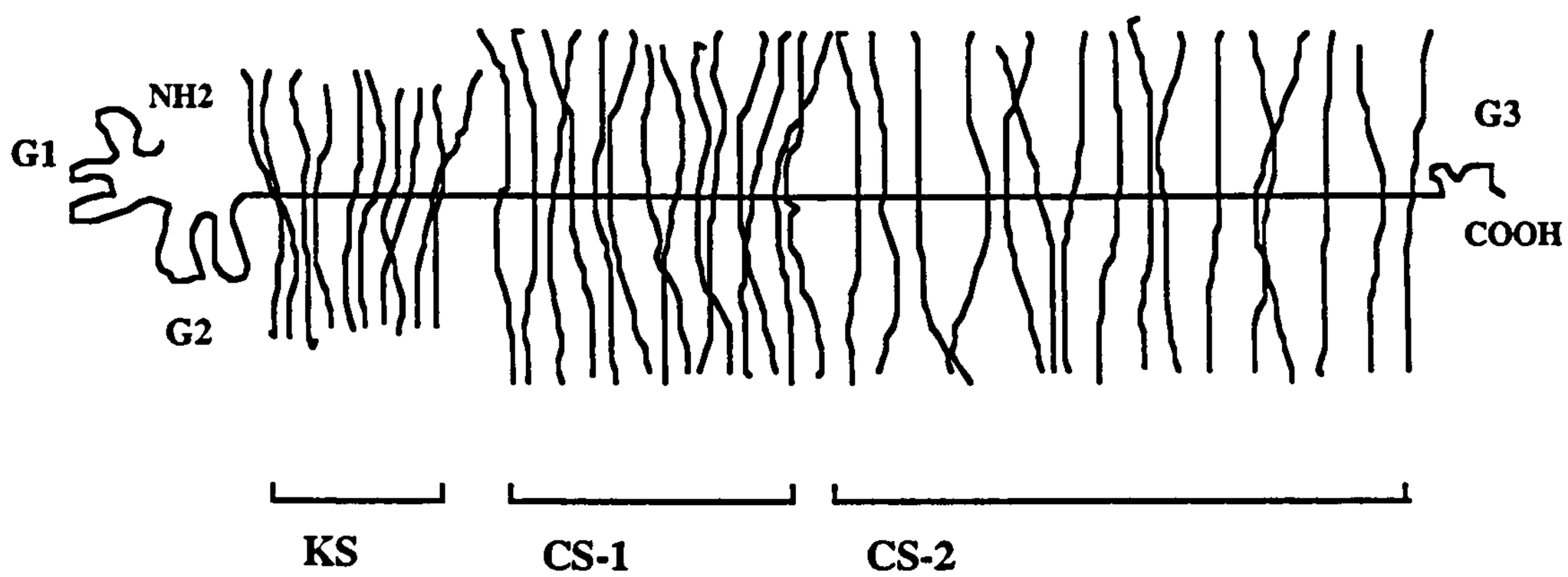

Figure 1-2: Aggrecan[2]

KS = Keratan sulphate CS = Chondroitin sulphate
G1, G2, G3 = globular domains (G1 = HA binding region)

[2] Adapted from Poole AR: Cartilage in health and disease. In McCarty DJ(ed): Arthritis and Allied Conditions. A textbook of Rheumatology. Philadelphia, Lea and Febiger, 1993,pp279-333

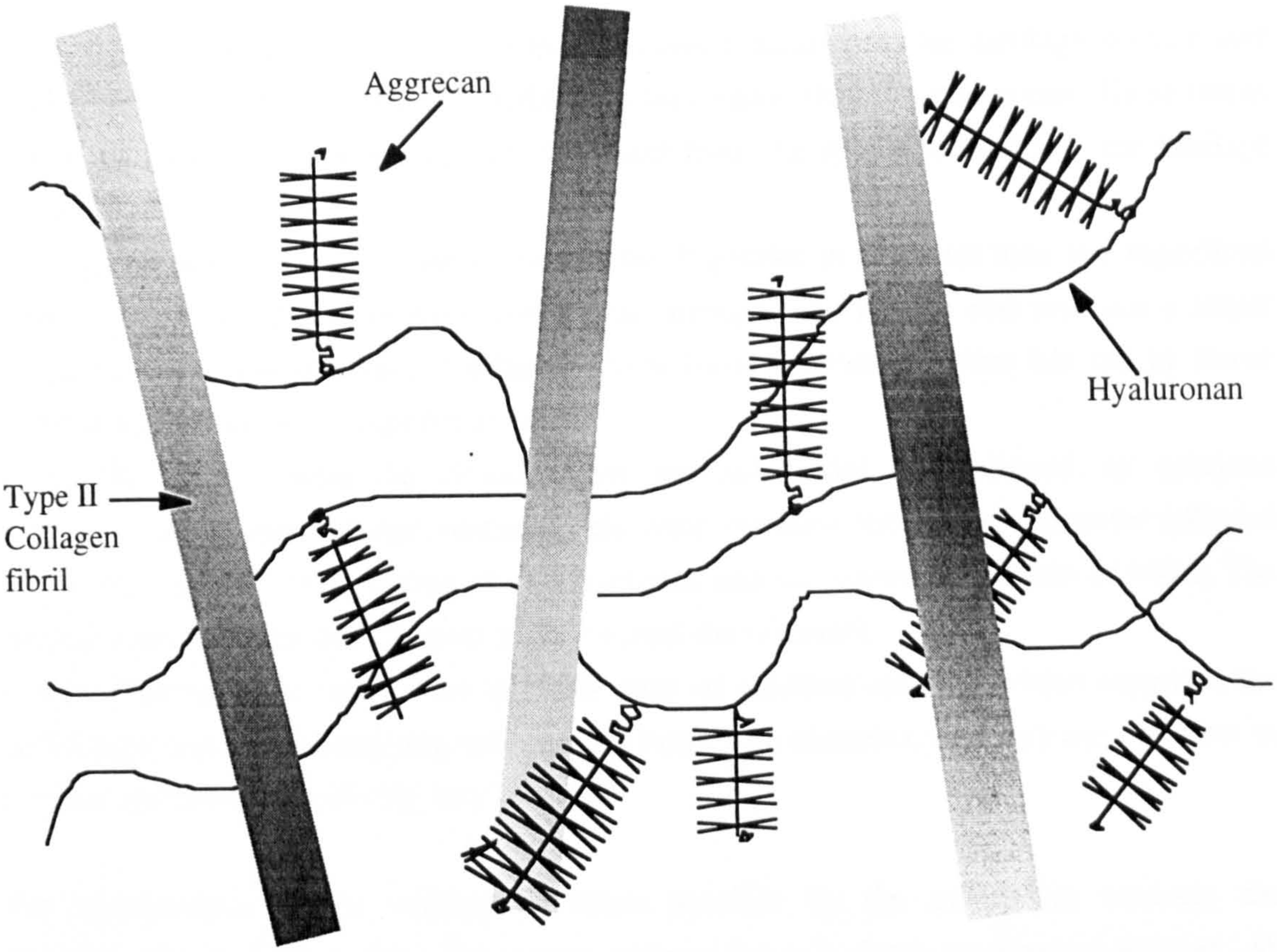

Figure 1-3: Organisation of type II collagen fibrils and proteoglycans within the extracellular matrix[3]

c) Non collagenous proteins

These proteins are less well understood. Anchorin CII may help to anchor chondrocytes to the collagen fibrils of the matrix (Mollenhauer *et al.* 1984), and cartilage oligomeric matrix protein (COMP) is concentrated mainly around the chondrocyte and appears to have the capacity to bind the cells (Dicesare *et al.* 1994). Fibronectin is found in many tissues as well as cartilage, and is thought to have a role in matrix organisation and cell - matrix interactions (Miller *et al.* 1984).

Cartilage zones

As previously mentioned the cartilage is arranged in four distinct zones:

1. The superficial zone - here the chondrocytes are ellipsoid and arranged so that their axes are parallel to the cartilage surface. In this zone these cells produce a high level of collagen and a low concentration of proteoglycans in proportion to other zones (Aydelotte

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[3] Adapted from Poole AR: Cartilage in health and disease. In McCarty DJ(ed): Arthritis and Allied Conditions. A textbook of Rheumatology. Philadelphia, Lea and Febiger, 1993,pp279-333

et al. 1996). Collagen fibrils are densely arranged parallel to the cartilage surface and impart greater tensile strength and stiffness to this region than to other zones. These fibrils also form a barrier to the passage of molecules from the synovial fluid into the cartilage matrix.

2. *Transitional zone* - this zone is usually much greater in diameter than the superficial zone. The chondrocytes are more spheroidal, arranged randomly, and produce a larger proportion of proteoglycans. Collagen fibrils have a wider diameter but are in lower concentration than in the superficial zone.

3. *Radial zone* - here the chondrocytes are spheroidal and aligned in columns perpendicular to the cartilage surface. This zone contains the largest diameter collagen fibrils, the highest concentration of proteoglycans and the lowest proportion of water. The interface between this and the next zone is called the tidemark.

4. *Calcified cartilage zone* - this is a thin zone of calcified cartilage which separates the radial zone from the underlying subchondral bone. The chondrocytes here are very low in number and are metabolically very inactive.

The maintenance of the cartilage is made possible by the interaction between the chondrocytes and the matrix. The matrix protects the cells from mechanical damage. All nutrients, newly synthesised molecules, waste products and regulatory molecules must pass through the matrix, and the rate at which this can occur depends upon the organisation of the matrix, primarily the concentration and composition of the large aggregating proteoglycans. Matrix macromolecules are constantly being synthesised and degraded by the chondrocytes throughout life, and this homeostasis is thought to be controlled by anabolic and catabolic regulatory factors i.e. cytokines. These cytokines are synthesised by the chondrocytes in response to a variety of stimuli, including mechanical loading. It seems that maintenance of the normal composition of cartilage requires a minimum level of loading and movement of the joint (Buckwalter and Mankin 1997).

2. Synovium

The synovium is modified mesenchyme which forms the innermost lining of the joint capsule. An incomplete layer of one to four synoviocytes form the intima, and these cells are both phagocytic (Type A or macrophages) and secretory (Type B or fibroblasts). The function of the synovium is to form a selective barrier for ultrafiltration of the plasma and thereby determine the composition of the synovial fluid . Small molecules less than 10kDa equilibrate between plasma and synovial fluid and cross the synovium by simple diffusion. Synovial cells synthesise hyaluronan which is secreted into the synovial fluid at the plasma membrane. It is also likely that lubricin, which is involved in the boundary lubrication of cartilage, is synthesised by the synovium (Swann 1982) .

3. Synovial fluid

Synovial fluid is a microfiltrate of plasma and fills the synovial cavity between the cartilage surfaces. It is a yellow, translucent, and viscous fluid, the latter property being the product of hyaluronan. It contains mononuclear cells, of which 90% are synovial lining cells, monocytes and lymphocytes. The function of the synovial fluid is to lubricate the articular surfaces of the joint and provide nutrients to the articular cartilage.

Hyaluronan

Hyaluronan is the only glycosaminoglycan which is non sulphated and does not have a protein core. It has a molecular weight of 1-2 million and in solution adopts a randomly coiled spherical configuration causing the large molecular domains to overlap and entangle, which imparts a high viscosity to the synovial fluid. This viscosity enables the synovial fluid to resist strain and to function as a boundary lubricant to the synovium. Hyaluronan also functions as a barrier to small molecular exchange.

4. Subchondral Bone

Subchondral bone supplies structural support to the overlying articular cartilage, and its stiffness enables it to support relatively high loads without substantial deformation. It is the principal shock absorber in the joint. The rigidity of the bone is a consequence of its high mineral content which is in the form of hydroxyapatite and makes up about 65% of the total bone matrix. 25% of bone is water and the remaining dry weight is made up of 95% (predominantly Type I) collagen and 5% proteoglycans and other noncollagenous proteins. Remodelling of the subchondral plate and the epiphyseal bone is under the control of the bone cells, the osteoblasts and the osteoclasts. Osteoblasts are mainly bone forming, are situated on the bone surface and secrete the extracellular matrix or osteoid. Once surrounded by osteoid they are known as osteocytes. Osteoclasts are large multinucleated cells which resorb bone.

Pathogenesis of Osteoarthritis

Since the ordinal feature of OA is considered to be focal loss of articular cartilage, the pathogenesis of OA is mainly described in relation to this tissue. However it is important to remember that this disorder involves all the tissues of the joint and the processes change and vary in different tissues and in different joints. OA is not a static degenerative condition but also includes a high rate of synthetic activity. There is a mixture of degradative, reparative and inflammatory processes occurring in cartilage bone and synovium. The variations in the clinical presentation of OA are likely to reflect alterations in the balance of these processes (Dieppe 1991).

In the normal joint, synthesis and degradation of the cartilage matrix are controlled by the chondrocyte and continue in homeostasis throughout the healthy life of the cartilage . However in some instances the synthesis of new proteoglycan molecules becomes inadequate with a net result of proteoglycan loss. This in turn leads to water loss from the cartilage and reduction in the compressive strength.

There are three main stages in the process of cartilage degeneration (Buckwalter *et al.* 1997). In the first stage there is disruption of the macromolecular matrix framework, associated with an increase in water content , which may be caused by mechanical insult, degradation of matrix macromolecules, or disruption of chondrocyte metabolism. The collagen framework may be damaged, and proteoglycan aggregation and aggrecan concentration decreases. Chondrocytes respond in the second stage by proliferating and by increasing the synthesis of proteoglycan, which may be adequate to restore the defect or maintain it in an unaltered state for many years. However, the third stage occurs when this cell response fails and can no longer maintain adequate synthesis. Loss of articular cartilage follows.

Macroscopically the typical pattern of OA is characterised by progressive degenerative changes in the articular cartilage including yellowish discolouration, dullness, fibrillation, wearing or ulceration, and eburnation. The synovium and the fibrous layer of the joint capsule become thickened and congested. The first sign of disruption of subchondral bone in OA is increased density caused by formation of new bone on existing trabeculae. There is also the appearance of regenerating cartilage on the subchondral bone surface, most commonly at the joint periphery where osseous and cartilaginous excrescences can form osteophytes. The end stage of OA occurs when the articular cartilage has been completely lost and the articulating surface becomes dense subchondral bone. Bone remodelling and loss of articular cartilage result in altered joint shape which can lead to deformity and instability.

In the high motion equine joints OA changes are usually first seen grossly at the joint margins (McIlwraith 1982). Synovitis is frequently prominent, and there is a sequence of cartilage discolouration, fraying, erosion and ulceration in discrete areas. In low motion joints bony proliferation occurs with a tendency towards ankylosis in some cases. In OA of the distal intertarsal and tarsometatarsal joints subchondral bone lysis may occur, in contrast to the sclerosis which is a diagnostic feature of OA in most other joints (Morgan 1968).

Biochemical changes

Metalloproteinases

Enzymatic degradation of articular cartilage is thought to precede morphological breakdown and plays a central role in equine joint disease. The most well known group of matrix destructive enzymes are known as neutral metalloproteinases which include collagenase (MMP-1) stromelysin (MMP-3), and the gelatinases (MMP-2 and MMP-9). These enzymes are produced mainly by the chondrocytes and synoviocytes after stimulation by the cytokine interleukin-1 (IL-1) (Morris *et al.* 1994) (Mort *et al.* 1993). They are secreted as latent proenzymes and activated by serine proteases. Plasmin may activate stromelysin and stromelysin in turn activates collagenase. MMP-1 is capable of cleaving Types I, II, and III collagen into small fragments. MMP-3 has a wide range of substrate specificity and is therefore an important contributor to tissue destruction. It is involved in normal matrix turnover and in destruction by clearing the proteoglycan aggregate (Sandy *et al.* 1992) releasing large chondroitin sulphate fragments, and this in turn can potentiate the effect of MMP-1. MMP-2 degrades type IV collagen and may further degrade the collagen fragments produced by the action of MMP-1.

During normal matrix turnover there is no change in the size of glycosaminoglycan chains or in proteoglycan distribution. This may be because inhibitors in the local environment may be sufficient to block the action of the low levels of proteinases. However in the OA process following increases in the MMP levels , chain size changes do occur, implying inhibition of MMP inhibitors.

The major inhibitors of matrix metalloproteinases are the TIMPS, heavily glycosylated proteins that bind to the MMPs and inhibit their action. A deficiency of TIMP has recently been found in OA cartilage (Dean *et al.* 1987) and it is currently thought that the balance between MMPs and TIMPs is responsible for the rate of articular cartilage degeneration.

Cytokines

The metalloproteinases are activated by the action of mediators of inflammation known as cytokines. Activated macrophages, synoviocytes and connective tissue cells have all been shown to be capable of secretion of these inflammatory mediators, believed to be the primary determinants of articular damage after joint injury (Pelletier *et al.* 1993).

Interleukin (IL-1) has been isolated from the articular cartilage culture media and synovial fluid from many species (Morris *et al.* 1990). The function of IL-1 is the induction and

synthesis of the metalloproteinases and the inhibition of the cartilage extracellular molecules, collagen and the aggregating proteoglycans. The recent demonstration of naturally occurring inhibitors of IL-1 in different species implies an endogenous control of cytokine activity (Seckinger *et al.* 1990) and it is therefore likely that the balance between IL-1 inhibitors and active IL-1 determines the degree of the inflammatory response.

Other cytokines including tumour necrosis factor (TNFα), transforming growth factorβ (TGFβ), insulin-like growth factor (IGF-1) have also been shown to regulate the expression of metalloproteinases and TIMPS.

Role of mechanical factors

Mechanical forces can destroy cartilage indirectly by insult to subchondral bone, synovial membrane or chondrocytes. One concept (Radin *et al.* 1986) is that subchondral bone sclerosis causes a reduction in the shock absorbance of subchondral bone which then puts the cartilage at risk of tensile failure by damage to collagen crosslinks, particularly under repetitive impact loading. In the horse mechanical stresses are crucial in the pathogenesis of OA and use trauma is generally accepted to be a principal aetiological factor (see later). Different areas of cartilage are subjected to different types of loads which results in each area having a different inherent biomechanical capacity and biochemical composition (Palmer *et al.* 1996). Loading stimulates the synthesis of proteoglycan and increases chondrocyte metabolism and is essential for the maintenance of healthy cartilage matrix. However, high and continually compressive loads can be damaging to the articular cartilage since the level of increased proteoglycan synthesis cannot be maintained. There appears to be a critical level after which the beneficial effect of exercise is overwhelmed by the harmful effects of repetitive loading, a crucial consideration in the training of performance horses. During light training the increase in chondrocyte potential to synthesise proteoglycans results in adaptive remodelling which serves to protect the cartilage. However the mechanical stress often encountered in equine training can overwhelm the adaptive response and alter the biochemical composition of the matrix. Increasing or maintaining exercise at this level may result in irreversible changes to the joint tissues, thus continued mechanical loading is a major driving force for the development and progression of OA.

Classification of osteoarthritis

As in man , OA in the horse has been classified in many different ways.

Type 1	Acute - associated with synovitis and high motion joints
Type 2	Insidious - associated with low motion joints
Type 3	Incidental or non- progressive cartilage erosion
Type 4	Secondary to other identified problems e.g. a) Intraarticular fractures b) Dislocations/ligamentous rupture c) Wounds d) Septic arthritis e) Osteochondrosis
Type 5	Chondromalacia of the patella

Table 1-1 : Original classification of OA in the horse by McIlwraith (McIlwraith *et al.* 1988)

Type 1 is more common in the young racehorse and affects primarily the more mobile high motion joints e.g. the carpus and the metacarpo/tarsò phalangeal joints. Synovitis and capsulitis are very common and usually precede the osteoarthritic process.
Type 2 is usually insidious in onset and affects the low motion joints e.g. the interphalangeal and intertarsal joints. Type 3 describes lesions which may be identified at post-mortem examination but are of unknown clinical significance. Type 4 describes OA that develops secondarily to other joint injury and Type 5 refers to a condition similar to chondromalacia of the patella in man, involving fibrillation of the articular cartilage of the distal aspect of the articular surface of the patella.

However this classification has now been altered in light of recent considerations.

Type 1	Associated with capsulitis and synovitis (common in carpus, metacarpo/tarso phalangeal , distal tarsal and distal interphalangeal joints)
Type 2	Associated with and usually secondary to other identified injuries or problems
Type 3	Incidental or non progressive articular cartilage erosions

Table 1-2: Modified classification of OA in the horse (McIlwraith 1996)

The distinction between the original Type 1 and Type 2 categories seemed inappropriate since the synovitis and capsulitis which are part of both syndromes are associated with repetitive cyclic trauma. It was also felt that the contribution of primary pathological changes in the subchondral bone possibly leading to secondary articular cartilage damage needed to be recognised, as well as the failure of ligamentous adaptation to accommodate stresses to the cartilage.

Classification of OA in man used to include primary and secondary OA, but this is no longer considered applicable since OA is now thought to be a disease process secondary to possibly unknown factors, including biomechanical changes, local influences, and systemic factors. OA is now preferentially classified according to joint site, i.e. knee, hip, or digital OA, and to the site within the joint, e.g. medial OA of the knee (MOAK), lateral OA of the knee (LOAK) (Dieppe *et al.* 1994). It is interesting to note the similarity between this and the late 19th century method of classification of equine OA reviewed at the beginning of this chapter.

Epidemiology

There is a lack of literature concerning the epidemiology of equine osteoarthritis, and most of what is understood regarding the incidence of the disease is based on anecdotal evidence. This highlights an area of equine medicine which requires considerable investigation.

Aetiology of osteoarthritis

In equine studies, use trauma has long been accepted as the central aetiological concept (Mackay-Smith 1962). Conformation, inadequate conditioning and training regime, and improper shoeing are also definite aetiological factors. Chemical factors include release of chemical mediators by chronically irritated synovium. The initiating event may be mechanical or chemical and the two processes are closely inter-related, i.e. OA is caused by either an abnormal force on a normal joint or by a normal force on an abnormal joint (Figure 1- 5).

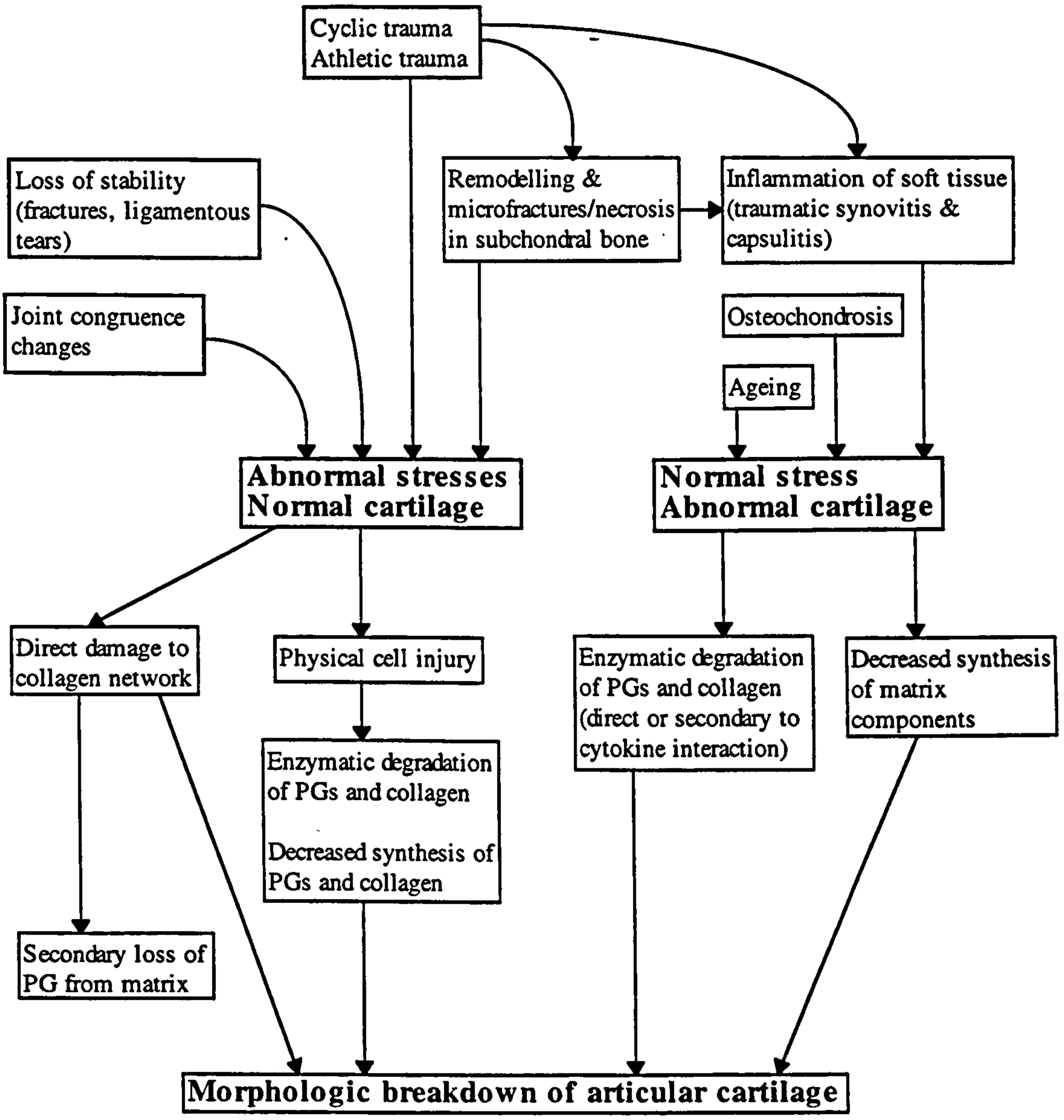

Figure 1-4: Factors involved in articular cartilage degradation in equine OA[4]

[4] Diagram from General Principles of Joint Pathobiology. In McIlwraith CW, Trotter GW(eds):Joint Disease in the Horse. Philadelphia, W.B.Saunders, 1996, pp42

Risk factors

Known risk factors for OA, taken mainly from human studies, vary according to joint site, and can be both general e.g. age, sex, and race, or localised, e.g. joint shape, trauma, or occupation.

1) Age - In man OA is age related but not caused by age. In the horse this is also true, although overall the relative age of equine patients is younger (McIlwraith 1996).

2) Sex and race - in man OA of certain joint sites has a predilection for different sexes, hip OA being common in caucasian males, while hand and knee OA is common in caucasian females (Dieppe 1991). There is no evidence in the literature to this effect in the horse.

3) Inherited susceptibility - although no single gene locus has been identified it is very likely that genetic predisposing factors are very important in man.

4) Obesity - Knee OA is very strongly related to obesity in man with the overweight having a seven times greater risk of knee OA development (Felson *et al.* 1995).

5) Trauma and selected activities - this certainly seems to be true in the horse (Table 1-1). In man certain joints have been found to be at higher risk of OA in certain activities e.g. wicket keeper's thumbs. However it has been found that normal athletic activity does not increase risk of OA without the existence of underlying joint abnormality (Dieppe 1991).

It is obvious that from the difficulty encountered in reaching an acceptable definition and classification of OA that there are many questions to be answered. If therapies are to be successful diagnosis must be made at an early stage and response to therapy must be monitored. Effective methods of measuring change in OA are vital for accurate trials of OA drugs. In order for prognoses to be made following trauma or disease, methods of predicting possible OA changes are fundamental.

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Chapter Two

Diagnosis , assessment, and therapy, in equine osteoarthritis

Aims of the thesis

Physicians of the utmost fame
Were called at once, but when they came
They answered, as they took their fees,
"There is no cure for this disease."

Hilaire Belloc 1870 - 1953

Diagnosis

1. Clinical signs and examination

Since radiographic features of joint disease correlate poorly with the clinical symptoms and stage of disease, the initial physical examination of the horse is an important part of the assessment of the disease. This should include a detailed history of the clinical signs including performance history, duration of lameness, previous injury and previous intra-articular therapies. Assessment of conformation should be followed by palpation of joints in both weight bearing and flexed positions to identify sites of heat pain and swelling. Crepitus originating from damaged cartilage surfaces or from soft tissues can be assessed by palpation, as can the degree of effusion the joint. Pain can originate from the fibrous joint capsule, the intra-articular ligaments, the subchondral bone , the periosteum , the synovium and the periarticular ligaments, and can be detected either by a painful withdrawal response on palpation or by an alteration in gait on exercise. A decreased range of motion of a joint is commonly found in OA and can result from pain, joint effusion or capsular fibrosis.

Lameness in OA is caused by pain, or mechanical restriction of the joint. The degree of lameness, or alteration in gait, is commonly the primary method of measurement of the degree of pain, or the extent of the disease process. However the degree of lameness does not vary directly with the extent of change observed on radiographs (Trotter *et al.* 1996),

and the type of gait is not necessarily associated with the type or origin of OA. Good correlation has been found in one study between soft tissue changes and degree of lameness but correlation between articular cartilage damage and lameness was poor (Trotter and McIlwraith 1996). Validation of lameness in the assessment of OA is necessary to evaluate its use as an outcome measure.

Regional and intra-articular anaesthesia are commonly used methods for localisation of lameness in the horse. Intra-articular anaesthesia is more specific in isolating the source of pain to the joint but false positive results may occur where regional nerves lie close to a synovial outpouching and similarly false negative results may be possible when the pathological lesion lies within the subchondral bone.

2. Radiography

Radiography is considered to be the primary method of OA diagnosis in man and horse (Spector *et al.* 1993; Widmer *et al.* 1994).

Radiographic features of OA in the horse

Subchondral bone sclerosis is usually a response to cartilage erosion but could also be the cause of cartilage degeneration. The increased thickness may be difficult to appreciate in the horse because most equine joints already have a naturally thick subchondral bone plate. Subchondral lysis is more common in the small tarsal joints than elsewhere. Lysis in OA is usually focal except in the tarsus where it can be seen extensively throughout the joint. Lytic areas with indistinct margins usually represent active lesions whereas those with well defined margins may be chronic and inactive. Periarticular osteophytes occur on the joint margins. These may be produced because of joint instability or stimulated by cartilage degradation products (Johnson 1962) and their relevance to the activity of OA has been much debated. Other periarticular bone changes include enthesiophytes which are the result of ligament or capsule tearing and ossification at the joint insertions. Articular cartilage changes are represented by narrowing of the joint space indicating cartilage erosion and degeneration. This narrowing may be generalised or focal and is best appreciated by comparison with the contralateral joint. However, joint width can be difficult to evaluate in the horse, since it is greatly affected by uneven weight bearing, which is a common problem in the standing horse.

Radiography is non invasive, fast, cheap and available but it is insensitive to early changes and can not therefore be used to identify OA at early stages of the disease, when interventions may have more chance of success. It is also not ideal in monitoring response

to therapies as changes occur only slowly. It can reflect only the degree of bone destruction, joint space narrowing and deformity that occurs and provides little information regarding soft tissue changes that occur. It has been shown to be poorly correlated to other measures of outcome. In equine literature there are few reports of reproducibility studies.
Radiography will be further discussed in Chapter 6.

3. Synovial fluid analysis.

Analysis of synovial fluid can provide valuable additional information but is not diagnostic of OA. Changes in visual appearance and increase in volume can indicate synovitis. Measurement of protein content is routinely done but is not particularly useful in OA. An increase in the protein content above 2.5g/dl is indicative of inflammation . Measurement of viscosity of synovial fluid gives an estimate of the quantity and degree of polymerisation of HA (Persson 1971) .The white cell count may be useful but varies tremendously in OA depending on the degree of active synovitis.

Studies have been carried out on the analysis of cartilage particles in the synovial fluid and a positive relationship was reported between cartilage debris and lesions on the articular cartilage surfaces (Tew 1980). However this technique has not been widely accepted since doubts were expressed concerning the presence of false results (McIlwraith 1980) .

4. Arthroscopy

Arthroscopy allows direct evaluation of non-osseous tissues including articular cartilage, synovium and intraarticular ligaments. However it requires an experienced operator and is an invasive technique necessitating general anaesthesia (McIlwraith 1978). Grading scales have been developed in both man and in horse for scoring cartilage lesions, but further studies have yet to be done on the validation of these scales. See Chapter 8.

Future methods of OA assessment

1. Scintigraphy

Scintigraphy has been shown to be more sensitive than plain radiography in detecting pathological changes associated with OA of the knee (Alzaraki *et al.* 1975) and is considered highly sensitive and specific for OA (Mooar *et al.* 1987). It is indicated in cases of multiple cause chronic lameness, evaluation of cases with painful yet radiographically normal joints, for evaluation of joints that cannot be radiographed in the

standing horse, and for evaluation of the treatment response (Devous *et al.* 1984). To date there have been no reports in the literature concerning the validity of this technique as a measure of outcome in OA. This will be further discussed in Chapter 6.

2. Other imaging methods

Ultrasonography has been used extensively in the horse for the evaluation of periarticular soft tissue structures, muscles, ligaments, joint capsule and tendons but imaging of internal anatomy of joints is more limited. Computed tomography is most usefully employed in conjunction with radiography and provides better definition of bone lesions. However the equipment necessary for this technique is not yet widely available.

Magnetic resonance imaging can provide exceptionally good images of articular cartilage and intra and periarticular soft tissue structures and is an extremely promising technique for diagnostic imaging in equine OA. However it is not readily available for horses *in vivo* and most studies to date have been carried out on cadaver specimens (Park *et al.* 1987).

3. Molecular markers of OA in synovial fluid and serum

During the dysregulation of the normal balance of degradation and repair within the joint various molecules are released into the synovial fluid (SF) and serum. Some of the molecules are indicators, or "markers", of degradation, some of synthesis, some of synovitis, and some of increased bone turnover and these can be used as tools to detect levels of degradation or repair. In man, the synovial fluid level of keratan sulphate (KS) epitope 5D4 is a putative marker of increased cartilage catabolism in early OA (Ratcliffe *et al.* 1994). The expression of the chondroitin sulphate (CS) anabolic epitopes 3B3- and 7D4 occurs in OA cartilage and SF but not in normal joints (Caterson *et al.* 1990) while the native CS epitope 846 is found in increased levels in OA SF (Rizkalla *et al.* 1992). A change in the sulphation pattern of chondroitin sulphate in the cartilage matrix can also indicate early OA changes (Sharif *et al.* 1996) .The levels of metalloproteinases in the SF can also be used as markers of cartilage degradation (Lohmander *et al.* 1993) while cartilage aggrecan catabolism by the as yet uncharacterised enzyme "aggrecanase" generates neoepitopes which can be detected by monoclonal antibodies (Hughes *et al.* 1992). Increasing levels of cartilage oligomeric matrix protein (COMP) in SF has been found to predict progression in OA (Sharif *et al.* 1995). The concentration and molecular weight of hyaluronic acid (HA) in SF is thought to be a marker of synovial inflammation (Dahl *et al.* 1985) while bone sialoprotein (BSP) (Saxne *et al.* 1995) and bone specific alkaline phosphatase (BAP) are markers of increased bone turnover.

The use of marker studies in understanding the pathogenesis of OA may be a very productive use of current marker technology, as well as in the diagnosis, prognosis and assessment of change in OA. The use of biochemical markers will be further discussed in Chapters 7,8, and 9.

Therapy

1. Rest and controlled exercise

The use of rest, alone, or combined with other therapies, has been the traditional form of treatment for equine OA for many years. The amount, and form of rest recommended is variable, depending on the extent of the disability and on the opinion of the clinician. It may commonly begin with a period of stable confinement, followed by gentle controlled exercise, i.e. walking in hand. This generally progresses to turning the horse out in a field to rest further before returning to active training. The economics involved with horse ownership commonly prevents the proper application of rest therapy. This reflects the need for increased owner education in the cost /benefit ratio of this method.

2. Non steroidal anti-inflammatory drugs

Phenylbutazone has been used to treat lameness in the horse for more than 30 years (Lees *et al.* 1983). Other non steroidal anti-inflammatories (NSAIDs) used in lameness cases include flunixin meglumine, meclofenamic acid, and carprofen, but data concerning their effects on synovial metabolism in horses is lacking. All NSAIDs, to varying degrees, inhibit the action of cyclooxygenase, thereby interfering with the arachidonic acid cascade (Vane 1971). Another important action of NSAIDs is their effect on proteoglycan synthesis since in addition to inflammatory cascades, some NSAIDs (Muir *et al.* 1988) have been reported to affect cartilage anabolism, inhibiting proteoglycan synthesis.
Although there is no doubt that the NSAIDs are useful in the reduction of inflammation, more investigations are necessary into the effects of these drugs on osteoarthritic tissues. There is also a need to demonstrate the comparative efficacy of the various available NSAIDs in clinical cases of equine OA.

3. Intra-articular medication

a) Corticosteroids

The use of corticosteroids (CSD) intra-articularly in the horse was first reported in 1955 (Wheat 1955) and has now achieved widespread acceptance. They are potent anti-inflammatory drugs and will reduce synovitis and the concentration of deleterious

enzymes in the joint. However, intra-articular CSD therapy was first reported to induce arthropathy in 1968 (O'Connor 1968) and their use continues to be controversial, the relative risk to benefit ratio not yet having been agreed (McIlwraith 1992).

Little objective information exists regarding corticosteroid effects and therefore numerous investigations have been made recently to try to evaluate the physiological effects of corticosteroids on equine joint tissues (Chunekamrai *et al.* 1989; May *et al.* 1988; Trotter *et al.* 1991). Reports from controlled clinical trials concerning their therapeutic use are few, however in one recent placebo controlled trial the corticosteroid triamcinolone was reported to have beneficial effects on degree of lameness, and on both the synovium and articular cartilage, in horses with carpal osteochondral fragmentation (Frisbie 1997).

b) Sodium Hyaluronate

Intra-articular sodium hyaluronate (SHA) is useful in the treatment of synovitis and capsulitis although the exact mode of action is not clear. There is experimental evidence in the literature that the use of this drug improves the clinical symptoms of joint disease, but beneficial effects on cartilage lesions have not been observed (Wigren *et al.* 1978). Clinical trials have indicated that horses could return to work following a single SHA injection, but these trials have not been double blind or placebo controlled (Phillips 1989; Ruth *et al.* 1985). A study carried out in 1995 that was placebo controlled however did demonstrate a significant improvement in lameness in horses treated with SHA (Gaustad *et al.* 1995) but the duration of this trial was only 7 weeks.

The advantage of using a combination of SHA and CS for the treatment of equine OA was demonstrated first in 1970 (Rydell *et al.* 1970). More recently one study has suggested that the addition of SHA reduces the biochemical degradation that occurs with repeated CS therapy (Roneus *et al.* 1993).

In a recent placebo controlled trial (Kawcak *et al* 1997), the use of intravenous SHA was found to improve lameness scores and reduce synovial pathology in horses with experimentally induced articular cartilage defects , but had no effect on markers of cartilage pathology.

In conclusion, the use of SHA seems to be helpful in cases of mild synovitis and capsulitis but if articular cartilage degeneration is present, the results are unpredictable.

c) Polysulphated glycosaminoglycan

Polysulphated glycosaminoglycan (PSGAG) is a mixture of highly sulphated glycosaminoglycans, the major component being chondroitin sulphate. It has been reported to have an anabolic effect on synovial fibroblasts and chondrocytes in vitro causing a marked increase in HA synthesis (Smith *et al.* 1986), and also to stimulate net collagen and glycosaminoglycan synthesis in both normal and osteoarthritic equine metacarpophalangeal cartilage tisses in organ culture (Glade 1990). PSGAG has been

metacarpophalangeal joint by intra-articular injection (May *et al.* 1988). However, few properly controlled double blind clinical trials have been carried out in horses. In 1990 in a trial of 109 horses, decreased lameness, synovial fluid protein content, and improved clinical signs were seen more frequently in the PSGAG treated group (Hamm *et al.* 1988) and in 1995 a trial reported by Gaustad (Gaustad and Larsen 1995) proved PSGAG to significantly reduce lameness score when compared with a placebo. In both trials cases were only followed for up to 7 weeks.

PSGAG is a commonly used drug in the treatment of equine OA and because of its suspected chondroprotective properties is traditionally favoured where articular cartilage damage is suspected. Despite anecdotal reports of good clinical efficacy, laboratory investigations have produced conflicting reports, therefore more extensive studies on the effects of PSGAG on joint tissues, along with well constructed clinical trials are necessary.

4) Pentosan polysulphate

Pentosan polysulphate has been used as an antithrombotic-antilipaemic agent in Europe for many years, but its potential as a disease modifying drug in OA has only begun to be investigated in recent years. The functions and mode of action of pentosan polysulphate will be discussed further in Chapter 3.

5. Surgical treatment

Surgical treatment cannot reverse articular cartilage damage but may be done to re-establish pain free weight bearing in order to salvage a horse for non athletic pursuits. The most common surgical procedures include bone fragment removal, debridement of an articular cartilage surface defect and arthrodesis. Many techniques have been evaluated for promoting articular cartilage resurfacing. Recent studies indicate that while full thickness debridement and subchondral bone drilling increase blood supply and oxygenation of chondrocytes, it is most beneficial to preserve the subchondral bone plate, and this can be achieved by a new microfracture technique (McIlwraith 1997).

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Aims of the study

It seems that despite the common and debilitating nature of OA in man and in horse, the ability to make early diagnoses and to measure and predict changes still eludes us. Although many potential therapies have been investigated, there remains no efficient and reliable treatment for OA in the horse. This may be due, in part, to the lack of well designed and conducted clinical trials. Therefore there exists a need both for well run clinical trials to accurately assess new therapies, and a need for the early diagnosis of OA in order to treat OA before it becomes irreversible. Central to both these requirements is the need for valid and reliable methods of measuring outcome. This study investigates methods of conducting clinical trials in horses, identifies potential areas of difficulty and sources of variability, and assesses the validity of measures of outcome in OA.

The aims of the study were as follows:

1. To carry out a controlled, blinded, pilot clinical trial in order to assess the validity and reliability of the present commonly used measures of outcome in the horse, i.e. functional outcome and radiography, and the more recent scintigraphic and molecular marker techniques, as methods of measuring changes in OA occurring in horses in clinical trials, and to evaluate a potential disease modifying drug in osteoarthritis .

2. To establish normal parameters of molecular markers in equine serum and in synovial fluid from different joints.

3. To investigate the use of molecular marker technology to assess change in joint status and disease process both cross sectionally and in longitudinal change in OA.

4. To investigate whether there is variation in the metabolism of different normal equine joints, by the examination of response of the articular cartilage to challenge .

Chapter Three

Calcium pentosan polysulphate pharmacokinetics study

Introduction

Calcium pentosan polysulphate (CaPPS) is a potential disease modifying drug (DMOAD) in osteoarthritis (OA) and was the drug chosen for investigation in the clinical trial. The putative actions of this drug and the experimental evidence for these actions are described below. The purpose of this study was to ascertain whether CaPPS was reaching the joint in adequate concentration to perform these functions.

Sodium pentosan polysulphate (NaPPS) has been available for over 30 years as an anti - thrombotic/anti-lipaemic agent (Ghosh *et al.* 1992). Only relatively recently has its potential as a disease modifying agent in osteoarthritis been recognised and the calcium derivative of the salt developed. Pentosan polysulphate (PPS) is isolated from beechwood hemicellulose. It consists of repeating units of (1-4) -linked β -D -xylanopyranoses in which α -D -4 - methylglucopyranosyluronic acid residues are attached glycosidically at position 2 of every ninth xylopyranose ring. PPS has no direct analgesic properties but is said to provide pain relief by acting on the disease processes within the joint that cause the pain. It is therefore classed as a DMOAD and is known to have the following effects :

1. Cartilage

PPS has been found to have an affinity for cartilage. Using radioactive PPS a peak concentration of 4µg/g cartilage was found after a 3mg/kg injection, and therapeutic concentrations were maintained for up to four days post injection (Collier *et al.*).

a) Chondrocytes

PPS has been shown to have anabolic effects on chondrocytes. Using an *in vitro* lapine model where chondrocyte injury had been induced by brief exposure to sodium iodoacetate, NaPPS, at a concentration range of 10 - 200µg/ml, improved proteoglycan incorporation into the extracellular matrix (Cotesque *et al.* 1986). In another study, using primary lapine chondrocyte monolayers NaPPS consistently stimulated proteoglycan synthesis over the concentration range 0.1 - 10.0 µg/ml, and stimulation of 25% over control levels occurred at

1.0µg/ml (Collier *et al.* 1989). The concentration of PPS achieved in cartilage after intramuscular injection of the recommended therapeutic dose (3mg/kg) has been found to be approximately 1.0 - 1.5µg/ml (Burkhardt *et al.* 1986), therefore, according to these figures, the required concentration for stimulation of increased proteoglycan synthesis could be achieved *in vivo*.

b) Metalloproteinases

PPS is said to inhibit the actions of matrix metalloproteinases (MMPs) and to stimulate the synthesis of tissue inhibitors of metalloproteinases (TIMPs). At concentrations of 0.5 - 5.0 µg/ml PPS has been found to inhibit both rat and human fibroblast stromelysin (MMP-3) (Nethery *et al.* 1992). In a recent anterior cruciate ligament transection dog model of OA a significant reduction of active MMP-3 levels along with an increase in TIMP in the articular cartilage, was observed in dogs treated with 2mg/kg PPS IM weekly, as compared to the non treated control group (Rogachefsky *et al.* 1993).

2. Synovium

Studies have also shown PPS to stimulate the production of hyaluronan by synoviocytes. Maximum stimulation was obtained, using cultured synoviocytes from OA joints, at PPS concentrations of 0.25µg/ml but occurred between the concentration range 0.1 - 1.0µg/ml. The HA produced was also of larger hydrodynamic size than in non treated controls (Hutalidok *et al.* 1988). The rat air pouch model of inflammation was used to confirm these *in vitro* effects *in vivo* (Francis *et al.* 1993).

3. Periarticular tissues

PPS stimulates the release of tissue plasminogen activating factor, and suppresses the release of tumour necrosis factor alpha from activated monocytes which in turn decreases the release of plasminogen activator inhibitor (Klocking *et al.* 1986). These effects thereby increase fibrinolysis which reduces periarticular and subchondral bone thrombosis. In canine studies, using PPS at a concentration of 3mg/kg, clearing of plasma lipids was demonstrated by the increase in plasma lipase (Brunaud 1967). This has the potential to increase joint perfusion by the removal of lipid from microcapillaries.

Toxicity

PPS has been found to have very low toxicity - in mouse and rat the LD 50 of NaPPS and CaPPS given intravenously is > 600mg/kg and administered by mouth exceeds 6g/kg (Klocking *et al.* 1991).

Dosage

In canine studies the dose rate of 3mg/kg NaPPS was found to be optimal with the dosages of 1 mg/kg and 5 mg/kg being less effective. The improvement in functional outcome demonstrated in treated dogs after four weekly injections was shown to be maintained for four weeks after the final injection (Read *et al.* 1996). In the horse an optimal dose rate of 2mg/kg CaPPS has been suggested.

Development of CaPPS

The calcium derivative of pentosan polysulphate (CaPPS) has been found to be more easily absorbed after oral administration than the sodium salt (Klocking and Markwardt 1986). After a single oral dose of 5mg/kg the absorption of NaPPS after 1 hour was 1% while the blood levels of CaPPS were maintained for > 4 hours indicating 10 - 20 % absorption. This increased absorption of CaPPS relative to NaPPS, has been confirmed in unpublished studies in rabbits, dogs, and humans, using subcutaneous as well as oral routes. In man, peak blood concentrations of NaPPS (3µg/ml)have been found to occur 2 hours after intramuscular or subcutaneous administration of a 3mg/kg dose (Dawes *et al.* 1986; MacGregor *et al.*) while after a subcutaneous injection of 2mg/kg CaPPS a concentration peak of 5µg/ml occurs at 4 hours post injection (P. Ghosh - unpublished data).

The hypothesis that CaPPS, when administered intramuscularly, achieves a similar peak plasma concentration to that in man, i.e. 5µg/ml at 4 hours post injection, and can also reach the synovial fluid in therapeutic concentrations, was investigated by obtaining serial serum samples and a single synovial fluid sample from horses following intramuscular CaPPS injection.

Aims

The aim of this study was to investigate the pharmacokinetics of CaPPS in the horse after intramuscular injection, and to establish that CaPPS was reaching the joint in adequate therapeutic concentration after intramuscular injection.

Methods

Six horses, four female and two male, of mixed breed, and of mean age 10.2 years (range: 4 - 20 years), were used for this study (Table 3-1). All were clinically in good health and were free from locomotor disorders. Each horse had haematological and biochemical profiles within normal limits for our laboratory. All horses were stabled throughout the study. At time point 0, CaPPS at a dose rate of 2mg/kg was injected intramuscularly into the brisket. Serial blood samples were collected via indwelling jugular catheters at time points 0, 1 hour, 2 hours, 4 hours, 8 hours and 24 hours post injection. Synovial fluid was collected from the midcarpal joint of each horse under sterile conditions, 4 hours after CaPPS injection. Blood was collected into sodium citrate vacutainers (Vacutainer Becton, Dickinson). After centrifugation at 2000rpm for 5 minutes, plasma was removed and aliquotted into 0.5ml eppendorf tubes. Synovial fluid was centrifuged for 10 minutes at 4000rpm. to separate cells and the supernatant aliquotted similarly to plasma. 14 days after injection a further synovial fluid sample was collected by arthrocentesis from the midcarpal joint of each horse. The samples were stored at -20C until being sent in dry ice to the department of Professor P. Ghosh (Director of the Raymond Purves Bone and Joint Research Laboratories in Sydney Australia) for analysis.

Horse	Breed	Age	Sex	Weight
1	Arab	15	F	450kg
2	TB	20	F	500kg
3	TB	10	F	450kg
4	Polish pony	6	F	450kg
5	TBx	4	Mn	450kg
6	Belgian Warmblood	6	Mn	550kg

Table 3-1: Age distribution and breeds of horses used in CaPPS pharmacokinetics trial

Sample analysis

The samples were analysed for pentosan polysulphate using a monoclonal antibody 5B10 and 0-hour plasma spiked with CaPPS as standard in a validated inhibition ELISA (Kongtawelert *et al.* 1990) . The intraassay and interassay coefficients of variation were 4.2 +/- 2.8 and 16.7 +/- 13.8% respectively. All samples were assayed in duplicate and the mean value for each sample used in analysis.

Statistics

Results were normally distributed, so mean values and standard errors of the mean (SEM) were stated. Comparisons were made by the Students t test.

Results

Plasma

Time (hours)	Horse 1	Horse 2	Horse 3	Horse 4	Horse 5	Horse 6	Mean	SEM
1	0.945	0.697	0.615	1.183	0.969	0.586	**0.832**	**0.097**
2	1.029	1.051	1.328	1.568	0.970	0.802	**1.125**	**0.113**
4	1.891	0.872	1.606	2.812	1.747	1.240	**1.695**	**0.270**
8	1.428	0.434	1.128	1.421	0.940	0.566	**0.986**	**0.172**
24	0.249	0.139	0.000	0.000	0.260	0.163	**0.135**	**0.047**

Table 3-2: Plasma concentrations of CaPPS μg/ml

Time (hours)	1	2	4	8
2	0.037			
4	0.007	0.038		
8	0.280	0.351	0.005	
24	0.001	0.001	0.001	0.006

Table 3-3: Significance of changes in plasma CaPPS levels (Students t test)

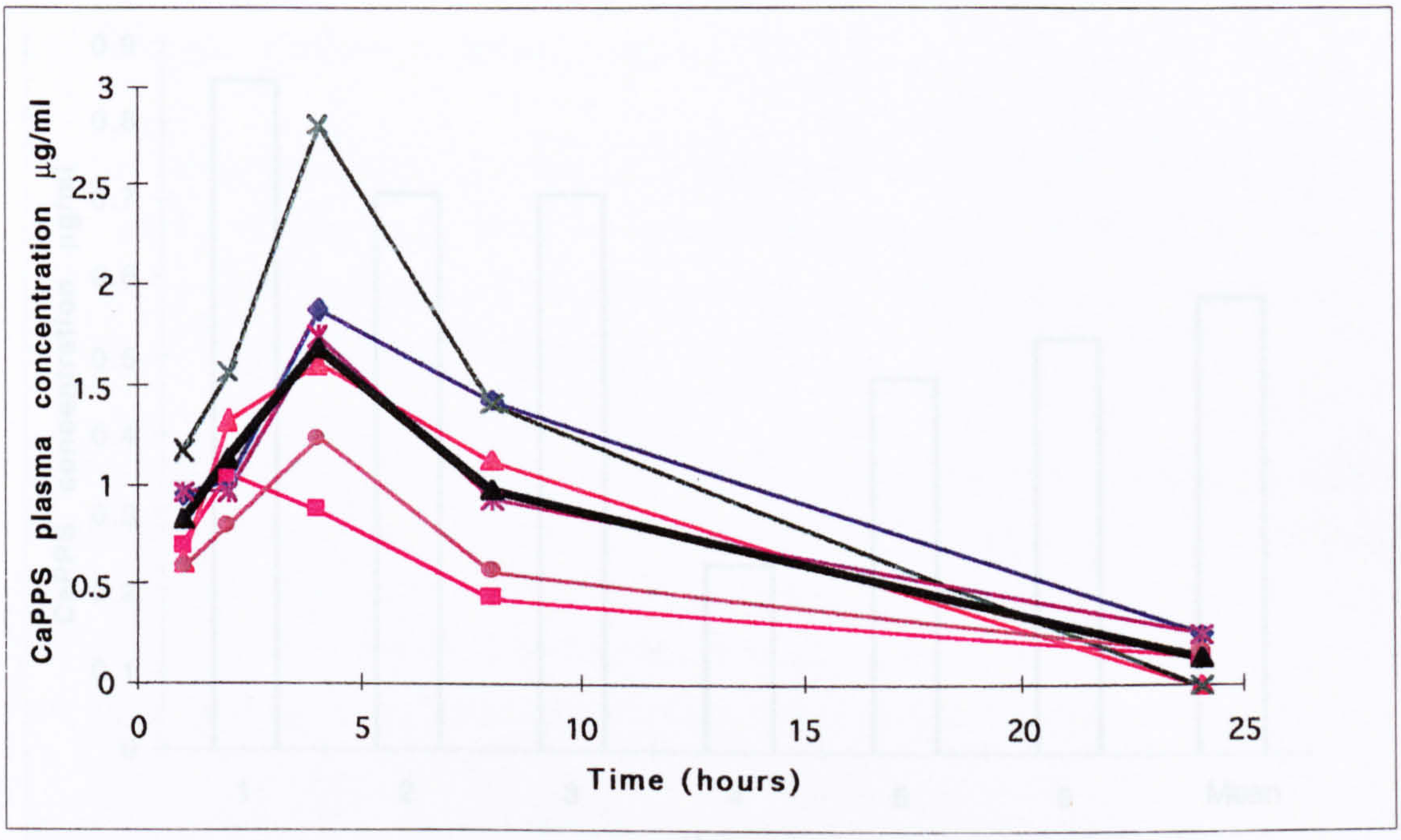

Figure 3-1: Mean levels of CaPPS in plasma after a single 2mg/kg intramuscular injection.

Overall mean level for all horses in bold line.

Synovial fluid

Time (hours)	Horse 1	Horse 2	Horse 3	Horse 4	Horse 5	Horse 6	Mean	SEM
4	0.852	0.712	0.711	0.238	0.480	0.530	**0.587**	**0.089**

Table 3-4: Mean levels of midcarpal joint synovial fluid CaPPS at 4 hours post intramuscular 2mg/kg injection

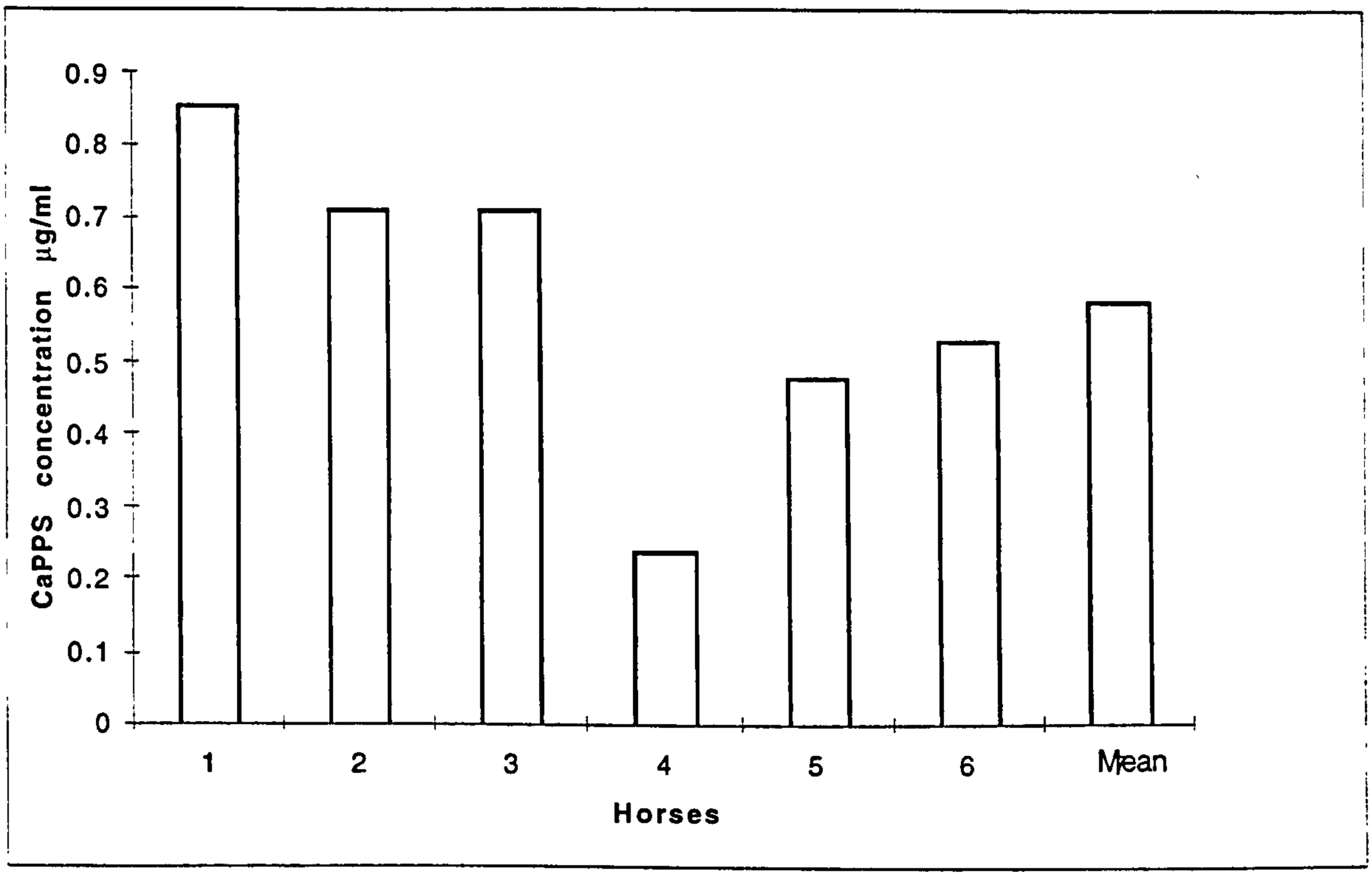

Figure 3-2: Synovial fluid CaPPS concentration for each horse, plus mean value at 4 hours post intramuscular injection.

Peak plasma levels of CaPPS occurred at 4 hours post drug administration. At this time, mean levels of 1.7µg/ml (range 0.9 - 2.8) were achieved in plasma (See Figure 3-1) and 0.6µg/ml (range 0.2 - 0.9) in synovial fluid (See Figure 3-2). There was no correlation between horse body weight and plasma CaPPS levels or between plasma CaPPS concentration at 4 hours and horse age.

Discussion

The mean peak concentration reaching the synovial fluid in this study was 0.6µg/ml. This is lower than the concentration of PPS found in cartilage by Burkhardt of 1.0-1.5µg/ml (Burkhardt and Ghosh 1986), and although the cartilage concentration was not measured here, it seems unlikely that it could reach this level since the major source of nutrition of the cartilage is from the synovial fluid. However, the CaPPS concentrations achieved here would

fall into the range reported by Collier as necessary to stimulate proteoglycan synthesis by chondrocytes (Collier and Ghosh 1989).

At the mean concentration of 0.6µg/ml achieved here the production of hyaluronan could be stimulated according to the study by Hutalidok (Hutalidok *et al.* 1988). The synovial fluid values measured here may not be maximum as the kinetics of transfer of CaPPS into the equine midcarpal joint are unknown.

Although it has been shown that a concentration of 0.5-50µg/ml is necessary to achieve 50% inhibition of rat and human stromelysin (MMP-3) , a decrease in activity of the enzyme has been reported at concentrations of 0.05 -0.5µg/ml (Nethery *et al.* 1992). It would seem therefore that the synovial fluid concentration of CaPPS achieved in this study would be adequate, potentially, to have an effect on the activity of these metalloproteinases.

Peak plasma levels of CaPPS were reached at 4 hours post injection, as in man. However, the peak concentration was only 1.7µg/ml (range 0.9 - 2.8 µg/ml) compared to 5µg/ml recorded in human pharmacokinetics studies (unpublished data). It is possible that this difference may be due to differences in elimination or excretion of CaPPS between man and horse but the mechanisms for these processes in horses are not known.

Summary

1. The levels of CaPPS found to reach the SF were in adequate concentration to elicit a potential therapeutic effect by action on the synoviocytes and by reduction in metalloproteinase activity. The concentration potentially reaching the cartilage would probably be lower than previously reported although still within the therapeutic range for chondrocyte stimulation.

2. Peak plasma concentrations occurred at 4 hours post injection but at a lower concentration than that reported in man.

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Chapter Four

Pilot clinical trial to investigate the efficacy of calcium pentosan polysulphate in osteoarthritis in the horse

Introduction

The purpose of this chapter is to describe and discuss the planning and execution of a clinical trial. Results of the various outcome measures used to assess the efficacy of the drug, including scintigraphy and molecular markers, will be discussed in detail in the subsequent chapters.

A clinical trial is the preferred method to assess the effects of a chosen treatment on naturally occurring cases of a disease. The prospective randomised controlled trial (RCT) is the gold standard method for assessing any medical therapy, but a very low number of RCTs are reported in the veterinary literature. A selection of these will be reviewed following an outline of the ideal trial design.

Trial design

A clinical trial is a prospective study which plans to make inferences about treatment efficacy from a sample of representative patients (Lund *et al.* 1994). There are several essential components of a good clinical trial, each of which will be detailed below.

1. Reduction of bias

The ultimate aim of good trial design is to eliminate or minimise bias. The following methods can be employed to achieve this.

a) Control / Comparison

The use of a two groups, one treated, and the other untreated or provided with an alternative therapy for comparison is essential in reducing bias. However, it has been reported that only 3% of articles describing study designs used in veterinary medicine described controlled trials, the remaining being case reports (58%), observational studies (15%), uncontrolled clinical trials (9%), case series (8%) and experimental studies (7%) (Smith 1988). The cases

assigned to each of the groups should be as homogeneous as possible, and the management of each group during the trial should be identical.

b) Randomisation and blinding.

Allocation to treated/control groups should be done randomly in order to maintain a balance between the two groups in the distribution of known and unknown variables which may influence the outcome. Randomisation also prevents any potential bias in the provision of treatment by the investigator. However, if numbers in the trial are small, and known variables exist, stratification is a useful way of ordering the randomisation. This involves the randomisation of cases to treatment groups within strata based on these factors e.g. breed, sex, age, stage of disease (Lund *et al.* 1994).

The specific treatment assignment of each case should be unknown, ideally, to both the patient and the investigator i.e. it should be double blind, and each assessment should be made by the same investigator. In veterinary medicine, where the patient is obviously the animal, the owner should be blinded to treatment since they have some input towards outcome assessment.

c) Chronology

Comparisons between treatment groups should always be made concurrently in order to prevent bias occurring from making assessments of different populations at different times.

2. Protocol

In the design of a clinical trial it is imperative that a proper protocol should be prepared and followed.

a) Hypothesis

The study must have both a null hypothesis and a primary outcome measure that are clearly defined.

b) Numbers and duration

The numbers of cases necessary for a trial depends upon the power of the experiment , i.e. the reliability with which rejection or acceptance of the null hypothesis can be made. Calculation of power will be discussed in more detail in the section describing statistical methods. Power depends upon the variability and the accepted magnitude of change in the

primary outcome measure chosen, so it maybe necessary to perform an initial pilot study to generate this data. Duration of the trial should depend upon the characteristics of the drug used and the primary outcome measure chosen.

c) *Inclusion criteria*

The definition of inclusion criteria is critical and should be strictly adhered to. Specification of inclusion criteria affects the external validity of the study i.e. the extent to which the results of the study can be generalised to wider populations, therefore the study group must be representative of the population to which the results will be eventually applied (Lund *et al.* 1994). Chosen cases must also be relevant to the disease under study so only those that have the potential to benefit from the intervention and for whom there is a high probability of detecting the effects of the intervention should be enrolled. Diagnostic criteria for the condition investigated must therefore be specifically defined. Relaxation of inclusion criteria increases the potential both for generalisation of the results and for larger outcome measurement variance, thus affecting study validity (Budsberg 1997).

d) Instructions and consent

It is paramount that the trial protocol is carefully explained to each owner, especially if there is a 50% possibility that their animal may receive placebo, since this may help to reduce non compliance at a later stage. Once this understanding has been reached, forms consenting both to the trial and to the use of an unlicensed drug should be signed.

e) Drug preparation

Preparation of the drug/placebo must be considered carefully to avoid any observable differences in preparation which may thereby unblind the trial. Instructions for correct drug administration must be explained carefully to each owner and any reactions to the drug must be noted.

3. Assessing outcome

The type of drug being investigated largely dictates the choice of methodology for measuring outcome. Recently drugs used in the therapy of OA have been classified as quick or slow acting, and the slow acting as symptomatic slow acting drugs in osteoarthritis (SYSADOAs), or disease modifying osteoarthritis drugs (DMOADs) (Lequesne *et al.* 1994). SYSADOAs are defined as those drugs which improve pain and/or function with a delay of 1 - 2 months but with a persistent benefit of some duration after treatment discontinuation (Theiler *et al.*

1994) . In human trials, the primary outcome variable for drugs designed to alter symptoms is usually joint pain as recorded by the patient. This can be done on a Likert scale or on a 100mm Visual analogue scale (VAS). A validated Pain index can also be used e.g. the WOMAC (Bellamy *et al.* 1985), Health assessment questionnaire (HAQ) (Fries *et al.* 1980), or the Arthritis impact scale (AIMS) (Meenan *et al.* 1980). The definition of DMOADs in human OA is outlined as a therapy which prevents, retards or reverses the morphological cartilaginous lesions of OA as determined using *in vivo* studies in man. The most important method of measurement of this activity is therefore that which demonstrates anatomic variability i.e. radiography, MRI, or arthroscopy. Changes in molecular markers as measures of disease process (Dieppe 1995) are also more recently being used in the investigation of DMOADs. To date no agent has been proved to be a DMOAD in man.

Outcome measures should be explicit and clearly defined, well established and clinically relevant, and valid, reliable, and responsive to change, a valid method being one which provides a significant discrimination power to distinguish the outcome between a placebo and a drug-treated group (Theiler *et al.* 1994). The degree of magnitude of change that would be considered positive and the variance of the outcome measure in the normal population must be known. Clinical trials should include a core of validated measures (a single primary response variable is considered preferable) e.g. in human studies, pain, physical function, patient global assessment and radiography (Altman *et al.* 1996) are commonly used. Scintigraphy and biochemical markers can be used as additional measures but have not yet been validated. In veterinary medicine little has been found in the literature referring to the validation of non objective scoring systems despite the most common response measurement made in veterinary studies being the subjective evaluation of pain or dysfunction.

The duration of a trial intended to test a SYSADOA depends on the rate of onset of the drug, i.e. these trials may range from 4 weeks to several months. In order to evaluate DMOADs however, a longer period is necessary and it is recommended that this should be at least one year (Altman *et al.* 1996) .

4. Statistics

An estimation of sample size should always be made before the beginning of the trial.
This estimation is based upon a) α - the probability of a false positive result or Type I error ; b) β the probability of a false negative result or Type II error; c) the variability of the primary outcome measure and d) Δ the hypothesised treatment difference i.e. that difference between the treatment groups that would be clinically relevant to detect. (Lund *et al.* 1994).
i.e.

$$\text{Sample size} = \frac{\text{Variability} \times [\text{Constant } (\alpha,\beta)]^2}{\Delta^2}$$

Where $\alpha = 0.05$ and $\beta = 0.1$ the Constant = 10.5 (Pocock 1983)

A Type I error will therefore detect a difference between treatment groups when no difference exists, and a Type II error fails to demonstrate a difference when a true difference does exist. Estimates of variance and hypothesised treatment differences Δ are obtained by experience, and when this is not possible, pilot studies should be carried out. The homogeneity of the population, the within patient variability of the outcome measure and the standardisation of the measurement techniques can all affect the variance . Type I or II errors can therefore occur when the sample size is inadequate, the outcome measurement is not validated, or when the measurement chosen is not appropriate to the drug being tested.
Power (1-β) indicates the ability of the trial at the given sample size and α level to detect differences between groups of a specific magnitude and its calculation is important in the assessment of clinical and statistical significance.
Although these calculations are rarely reported in clinical trials, in any trial where there is a possibility of a Type II error, an estimate of power and 95% confidence intervals should be calculated.
As well as presenting results of significance testing, the difference between treatment groups with the 95% confidence intervals of the difference should be stated (Ratain *et al.* 1990). Confidence intervals provide the range of values for which the result is thought to be true, enabling the magnitude of the true treatment difference to be estimated.
In any clinical trial the level of significance should be less than 5%, and the power more than 80%.

Previous clinical studies

There are very few reports in the veterinary literature describing clinical trials in equine OA, most OA studies in the horse using experimental ponies with experimentally induced OA. Reports do exist describing the treatment of horses with sodium hyaluronate (SHA) and polysulphated glycosaminoglycans (PSGAG). In 1976 Asheim and Lindblad (Asheim 1984) performed a clinical trial to investigate the effect of intra-articular SHA in racehorses. 45 horses with OA of the carpal, metacarpophalangeal, or distal interphalangeal joints were enrolled to the study. Diagnosis of OA was based on scoring the following factors - initial lameness, capsule thickening, increase in synovia, and radiographic evidence of osteophytosis. All horses had previously been treated with other methods but there was no mention of duration of lameness at the commencement of the trial. Some horses were given more than one course of treatment during the trial. A non subjective (but non validated) outcome measure was chosen, which involved the use of a scoring system based on the horse's racing capacity after treatment, by a race track veterinary surgeon. There was no mention of the number of different veterinary surgeons involved in this scoring however. The duration of the trial was adequate, being at least one year , but since there was no control group and no statistical analysis this study should be better described as a case series only, from which no definite conclusions can be drawn. In 1988 a multicentre randomised double blind clinical trial was carried out by Aviad (Aviad *et al.* 1988) to compare the effectiveness of two different molecular weight intra-articular hyaluronan products. Inclusion criteria are clearly described including lameness duration, and demographic data are clearly reported. Outcome measures included scoring of lameness, and joint heat, and measurement of joint circumference and synovial fluid protein content and viscosity. Sixty-nine horses were recruited to the trial but since this occurred at three different centres, different veterinary surgeons were employed in the evaluations which could have increased the variability of the already subjective scoring systems of lameness and joint heat. Statistical methods are well described but there is no calculation of power despite the fact that no significant differences were found and no use of confidence intervals. The trial duration was only 2 weeks.

In 1989 the trial carried out by Phillips (Phillips 1989) compared five different molecular weight hyaluronan products. 150 horses in athletic training were selected, and inclusion and exclusion criteria were clearly stated. Evaluation was blinded but the outcome measure although clinically relevant was again unvalidated - an overall assessment of return to active training or racing. Duration of the trial was 6 months. No explanation of statistical methods was given. The most comprehensive report of a clinical study found in the literature was by Gaustad (Gaustad *et al.* 1995) which was also the only one describing the use of placebo. A randomised double blind and placebo controlled clinical study was carried out to compare

PSGAG and SHA with placebo in the treatment of traumatic arthritis. Seventy-seven 3-4 year old standardbred trotters with moderate to severe lameness were recruited and randomly assigned to one of three groups. Randomisation was stratified with the number of affected joints per horse used as the stratification factor, however carpal, tarsocrural, metacarpo/metatarsophalangeal and distal interphalangeal joints were included in the trial and although all these were all classified as high motion joints no allowance for matching of these joints was made in the stratification. The method of diagnosis of arthritis and exclusion criteria was clearly stated. The study was carried out at one centre with one blinded assessor but the only outcome measure was a lameness score. Statistical methods are very clearly and comprehensively described but there are no calculations of power. The short 5 - 7 week duration of the trial is noted in the discussion of the paper but is justified by the explanation that in this time period some effect of the treatment is expected. The evaluation method is also noted as being subjective but the justification given for this is that the method is well established.

Overall the most striking observations made from the review of these reports is the common use of non validated outcome measures, particularly lameness scoring, the rare use of placebo controls, the short duration of the trials and the lack of calculation of power of the studies.

Pilot studies

Pilot studies, involving smaller numbers of patients, should be carried out before commencing full scale clinical trials. These enable the suitability of the protocol to be tested, and highlight any potential problems. Preliminary information regarding the biological effect of the drug can be gained, and the choice of outcome measures can be verified. Validity and reliability of these measures can be assessed if necessary and calculations of sample size necessary to provide adequate power can be based on pilot study results.

Trials investigating the use of CaPPS

Clinical studies in man.

In a non controlled study, 23 patients suffering from OA of the hips, knees or fingers were given five weekly subcutaneous injections of 2mg/kg CaPPS. Symptoms of pain, and the consumption of NSAIDs were reduced, and functional scores increased for up to 12 weeks post treatment (Verbruggen *et al.* 1994) . Another study of 105 OA patients, which was placebo controlled and double blind, also demonstrated a significant improvement in functional outcome for up to 3 months post treatment (Edelman *et al.* 1994).

Clinical studies in the dog

A double blind placebo controlled trial has also been used to demonstrate the efficacy of NaPPS in the dog (Read *et al.* 1996). 40 dogs which had been diagnosed with OA were randomly placed in one of four dosage groups i.e. 0,1,3, or 5 mg/kg dosage which was administered intramuscularly once weekly for four weeks. This was a multicentre trial and assessment was made, by a number of different assessors, by functional outcome scoring stiffness, mobility, pain on joint manipulation and overall response. Significant differences were reported between treatment groups, and the study concluded that 3mg/kg was the optimal dosage.

Clinical studies in the horse

There are no published clinical trials, but Sodium pentosan polysulphate (NaPPS) "Cartrophen Vet" Biopharm Australia is used in Australia for treating OA in horses. Intramuscular NaPPS has been used in management of chronic OA in 20 Thoroughbreds (Little *et al.* 1996) . There has been no critical analysis of results but anecdotal evidence suggests a beneficial effect. Treatment of these horses, with 2-3mg/kg NaPPS intramuscularly once weekly for four weeks, improved but did not eliminate the clinical signs including reduced synovial effusion and lameness. However, the most notable finding was reduction in increased lameness post racing. These horses could therefore resume training sooner and the interval between races was reduced.

Calcium pentosan polysulphate

Calcium pentosan polysulphate (CaPPS) was chosen for several reasons:-

1. It is a potential disease modifying drug.
2. There have been no clinical trials to date in the horse.
3. It is easy to administer by the owner by intramuscular route.
4. There is minimal risk of reaction (compared with intra-articular medications).
5. As a relatively new drug in therapy of OA it is appealing to owners.

The mode of action of CaPPS is described in Chapter 3.

Aims

The aims of this pilot clinical trial were as follows :-

1. To assess the validity, reliability, and responsiveness of lameness scoring and questionnaires as functional outcome measures in OA in the horse.

2. To evaluate radiography, scintigraphy and molecular markers as methods of assessing structural and disease modification in OA in the horse.

3. To make preliminary assessments on the use of a potential disease modifying osteoarthritis drug in clinical cases of OA.

Methods

Trial design

1. Reduction of bias

Control

It was decided that this trial should be carried out in a double blind, high dose/low dose controlled manner. Initially the trial was intended to be placebo controlled, but considerable owner resistance was encountered to the possibility of placebo treatment therefore the protocol was altered so that the trial could be dose related with a high (2mg/kg) and low dose (0.5mg/kg) group.

Randomisation and blinding

Once a definitive diagnosis of OA had been made (for method see later), each horse was assigned randomly (by a person not involved with the trial) to either a low dose (0.5mg/kg CaPPS) group (A) or a high dose (2mg/kg CaPPS) group (B). This random allocation was performed in a stratified manner i.e. the numbers of cases of OA in particular joints was equal in each group. The dosage used in each group was blinded to both owner and assessor, and this code was not broken until the final horse had completed the trial.

Chronology

Horses were recruited over a 12 month period. Horses in each group were treated concurrently.

2. Protocol

Hypothesis

The null hypothesis stated that no significant difference in outcome between the two treatment groups would be demonstrated.

Numbers and duration

Time and numbers of horses recruited to the trial were limited by the period available for the study . This was intended to be a pilot study, so for these purposes it was decided to recruit 20 horses to be observed over a 9 month time interval.

Visit	**1** (0 months)	**2** (3 months)	**3** (6 months)	**4** (9 months)
Procedures	Clinical examination	Clinical examination	Clinical examination	Clinical examination
	Scintigraphy		Scintigraphy	
	Intra-articular analgesia			
	Radiography	Radiography	Radiography	Radiography
	Video of gait	Video of gait	Video of gait	Video of gait
	Serum and synovial fluid sampling	Synovial fluid and Serum sampling	Serum and synovial fluid sampling	Synovial fluid and Serum sampling
	Questionnaire			Questionnaire
	CaPPS provision	CaPPS provision	CaPPS provision	
CaPPS treatment	↑↑↑↑	↑↑↑↑	↑↑↑↑	

Table 4-1: Trial protocol

(↑ - indicates each CaPPS injection, given once weekly for the first 4 weeks of each 3 month period)

Inclusion criteria

Diagnosis of OA

Horses were recruited from a population of riding horses referred to the Department of Veterinary Science, University of Bristol. In order to achieve as accurate a diagnosis of OA as possible, recruits to the trial were required to have a positive result in two out of the three following diagnostic techniques i.e. scintigraphic bone scan, intra-articular analgesia, and radiography.

Clinical examination

At the first examination before final recruitment to the trial, each horse was given a full examination. This consisted of visual assessment, limb palpation, and gait observation at the walk, trot, and lunge on both soft and hard surfaces if necessary. A lameness score at each gait, on a scale of 1 -10 (Wyn-Jones 1988) was given by the same assessor (CF) on each occasion. After flexion tests, intra-articular analgesia was performed to identify more accurately the site of the lameness.

Intra-articular analgesia

Using strict sterile technique a 19/20G x 1 inch needle was introduced into the joint using recognised approaches to each particular joint (Stashak 1987; Wyn-Jones 1988) After withdrawal of synovial fluid, both for sampling and for verification that the needle was correctly inserted, 8 -15 mls of local anaesthetic (Mepivicaine - Upjohn), depending on the joint, was injected. After between 5 - 20 minutes , further gait evaluation enabled the result of the analgesia to be assessed. Significant alleviation of lameness verified that particular joint as the site of pain.

Radiography

Radiography was carried out on both the affected and the contralateral joint, using standard views for each particular joint . A positive result for OA was based on the presence of joint space narrowing, subchondral bone sclerosis, subchondral bone lysis and osteophytosis (Widmer *et al.* 1994) although the exact parameters used altered depending on the particular characteristics of OA occuring in different joints (See Chapter 6).

Scintigraphy

This procedure was carried out on all recruits at the first examination, either before intra-articular analgesia was performed, or at an interval of no less than 10 days post intra-articular injections. Each horse was injected with 10MBq/kg Technetium99m

diphosphonate. After an interval of 3 hours a bone phase scan was carried out on each limb. Results were recorded both on hard copy and on computer (Maxi - Link systems). A positive result was defined by increased uptake of radioactive isotope in the subchondral bone of the joint (McCrae *et al.* 1992) indicating increased subchondral bone turnover (See Chapter 6).

Instructions and consent

Details of the trial were discussed extensively with each owner who were then required to sign a form consenting to both the use of an unlicensed drug and to the involvement of their horse in an investigative trial.

Drug preparation

CaPPS was provided to the owners at each of the first three visits. All doses of the drug were provided in identical 10 ml glass bottles either labelled "A" (low dose) or "B" (high dose). The low dose bottles contained 0.25g CaPPS in 10ml saline, while the high dose bottles contained 1g CaPPS in 10ml saline. Owners were instructed to inject 10mls of the provided dose of CaPPS intramuscularly once weekly for four weeks. Each owner was instructed in the correct procedure for administering and storing the CaPPS assigned to them.

During the trial each horse was examined by the same assessor (CF) on four occasions at 3 monthly intervals over the 9 month period, (i.e. at 0, 3, 6, and 9 months post diagnosis). At each examination a full clinical and radiographical examination was performed, and serum and synovial fluid samples were collected. At the first and third visit a scintigraphical bone scan was carried out . The gait of each horse at the walk and trot was recorded on videotape at each examination using a camcorder. Owners were requested to fill in questionnaires describing their horse's lameness at the beginning and end of the trial (Table 4-1). Owners were also advised to maintain their horse in a regime of controlled exercise, ideally boxed, or kept in a small paddock, with regular walking in hand initially, increasing the exercise to ridden walking as the trial progressed. It was advised that no other therapeutic agents should be used during the course of the trial.

3. Assessment of outcome

Parameters chosen to measure outcome were as follows:

Measures of function/disability

a) Lameness scores.

Lameness at each gait was scored on a scale 1 - 10 by the same assessor (CF) at each examination. Video recordings of the gait of each horse at each examination were rearranged and blinded to date order, and these were also used to score lameness by the same assessor (CF). A global score of improvement over the duration of the trial was given for each horse based on both lameness scores given at the time of examination, and those derived from the date blinded video recordings (Table 4-2).

Lameness compared to beginning of trial	Global score
Worse	-1
Same	0
Improvement	1
Sound all gaits	2

Table 4-2 : Global scoring system for lameness improvement

b) Questionnaires

Owners were asked to complete two questionnaires, one based on a visual analogue and the other based on a Likert scale, at the beginning and end of the trial. The questionnaires were designed to enable each owner to grade, in their own opinion, the degree of disability of their animal. Each owner was given instruction as to how to fill in the questionnaires on each occasion, but were not allowed to view their original answers when completing the second questionnaire. Six months following the conclusion of the trial a further questionnaire was sent to each owner to investigate the progress of each horse since the end of the trial.

Measures of anatomical changes in joints

Radiography

Radiography was carried out as described previously and in Chapter 6. Radiographs were scored qualitatively by four independent veterinary radiologists. See Chapter 6.

Measures of disease process

a) Scintigraphy

Scintigraphy was performed as described above and further in Chapter 6. Scans were scored qualitatively by four independent veterinary radiologists, and quantitatively by measuring an activity index for regions of interest on the computer. See Chapter 6.

b) Biochemical markers

Serum and synovial fluid sampling

Serum

At each examination, a single blood sample was taken from each horse into a plain vacutainer (Becton Dickinson) , the time of this sampling being recorded. Once clotted, blood was centrifuged for 5 minutes at 3000rpm. The serum was then removed by pipette and stored in 0.5ml aliquots at -80°C until analysis.

Synovial fluid

Synovial fluid samples were collected at the time of intra-articular analgesia at the initial examination , and then on following examinations by arthrocentesis. The colour, viscosity, and volume of each of these samples were recorded, before centrifugation at 4000rpm for 10 minutes. The supernatant was removed by pipette and aliquotted before storage at -80°C until analysis.

4. Statistical analysis

All the statistical tests used in the analysis of data from the trial were non parametric and involved the use of matched pairs. A 5% or less level of significance and 95% confidence intervals of the difference between groups were stated.

The necessary sample size and the power of the study was calculated by using the assumption that if a group of horses with osteoarthritis were not treated with CaPPS but maintained on a similar exercise regime for 9 months, 50 % of these horses would show some improvement using the primary outcome variable of clinical lameness improvement. If it is then accepted that for the CaPPS treatment to be deemed successful more than 70% of the treated group should improve, the resulting necessary sample sizes are shown in Table 4-3.

Number of horses improving		
Controls	Treated	Sample size in each group
50%	70%	95
50%	80%	45
50%	90%	24

Table 4-3 : Necessary sample sizes to provide 80% power

From Table 4-3 it can be seen that the numbers of horses in this trial were too small to provide adequate power. In this trial, with 10 horses in each treatment group, the power of the study would only be 29% if the improvement expected in the treatment group was 90% compared to 50% in the controls. For this reason this clinical trial could only be assessed as a pilot study.

Low dose group - 0.5 mg/kg						High dose group - 2mg/kg					
Horse	Sex	Age	Breed	Duration (months)	Joint	Horse	Sex	Breed	Age	Duration (months)	Joint
1	M	11	IDx	6	TMT	2	M	TB	4	8	PIP
3	M	12	TB	2	PIP	4	M	Welsh Cob	13	3	DIP
7	M	13	Pony	4	MC	5	M	Dales Pony	21	2	TMT
8	F	8	TB	3	DIP	6	F	TBx	7	24	TMT
10	F	7	TB	6	PIP	9	F	ID	9	3	DIP
11	M	16	ID	3	DIP	12	F	Eventer	6	3	PIP
13	M	9	Hunter	24	TMT	14	M	TBx	11	1	PIP
16	M	8	Lusitano	1	DIP	15	M	TBx	5	4	MCP
18	F	10	TB x ID	18	PIP	17	M	Arab x	2	3	TMT
20	M	14	Welsh Cob	5	DIP	19	F	TBx	18	5	DIP

Table 4-4: Demographic data of horses recruited to the trial

Key : TMT = tarsometatarsal joint
MCP = metacarpophalangeal joint
MC = midcarpal joint
PIP = proximal interphalangeal joint
DIP = distal interphalangeal joint
TB = Thoroughbred
ID = Irish draught

Results

Demographic data

20 horses from those referred to the Department of Clinical Veterinary Science, University of Bristol for persistent lameness, that were diagnosed to be suffering from OA, were recruited to the study (Table 4-4). These were of mixed breed and mixed age, the mean age being 10.2 years and ranging from 4 - 21 years. The sex ratio was 13 male : 7 female. The mean duration of OA in the population was 6.4 months. In 4 horses two joint sites were affected (7 - mid carpal and antebrachiocarpal, 13 - tarsometatarsal and tarsocrural, 4 - distal interphalangeal and proximal interphalangeal, and 14 - distal interphalangeal and proximal interphalangeal) , but in all of these cases one of these joints was more clinically active at the start of the trial and was therefore chosen for follow up (Table 4-4).

Trial design

1. Reduction in bias

After stratification based on the joint affected these horses were randomly assigned to either the low dose CaPPS group (Group A) or the high dose group (Group B). In the Group A there were two cases of tarsometatarsal (TMT) joint OA, three of proximal interphalangeal joint OA, four distal interphalangeal joint and one of midcarpal joint OA (See Table 4-3). The mean age of horses in this group was 10.8 years (range 7 -16) and the male to female ratio 7:3. The mean duration of OA in this group was 7.2 months. In Group B there were three cases each of TMT OA, PIP OA and DIP OA. There was also one case of metacarpophalangeal (MCP) OA. The mean age in this group was 9.6 years (range 4 -18) and the male to female ratio 6:4. The mean duration of OA was 5.6 months. Both groups included a mixture of breeds . There was no significant difference between the age distribution nor between the disease duration of either group.

The total time taken to run the trial was 22 months. Horses from each group underwent treatment during this period.

2. Protocol

Acceptance of the protocol and owner compliance with the trial was good with only one horse (No 11) from group A being withdrawn from the trial after the preliminary examination because of the development of laminitis and another (No 6) not completing the trial after an

unrelated injury necessitated euthanasia before the final examination. Three owners were unwilling to bring their horses to the trial centre for the final examination, but were willing for this final examination to be carried out at their own premises. Therefore 18/20 horses completed the trial, and 19/20 were still included at the third (6 months) examination. The only investigative procedure that was not always successful was arthrocentesis. In two horses, (10 and 17) this was not possible for safety reasons! One owner (12) would only consent to the trial if no arthrocentesis was performed, however in this case regional analgesia was used as an alternative to localise the site of lameness. In all three cases (10, 12, and 17) since the diagnostic techniques of scintigraphy and radiography were both positive for OA the decision was made to recruit the horses despite the lack of intra-articular analgesia because of the difficulty in obtaining adequate numbers in the available time.

Low dose group					High dose group				
Horse	**Joint**	**IA**	**Scan**	**XRay**	**Horse**	**Joint**	**IA**	**Scan**	**XRay**
1	TMT	+	+	+	2	PIP	+	+	+
3	PIP	+	+	+	4	DIP	+	+	+
7	MC	+	+	+	5	TMT	+	+	-
8	DIP	+	+	-	6	TMT	+	+	+
10	PIP	-	+	+	9	DIP	+	-	+
11	DIP	+	+	+	12	PIP	ABSB	+	+
13	TMT	+	+	+	14	PIP	+	+	+
16	DIP	+	+	+	15	MCP	+	+	+
18	PIP	+	+	+	17	TMT	-	+	+
20	DIP	+	-	+	19	DIP	+	+	-

Table 4-5: Diagnostic criteria

Key : ABSB = abaxial sesamoid nerve block.

Inclusion criteria

17/20 (90%) of the horses included on the trial had a positive response to intra-articular analgesia (Table 4-5). One owner (12) refused this procedure so lameness was localised with peripheral neural analgesia, and in the two other cases (10 and 17) analgesia could not be performed in the hind PIP and TMT joints for safety reasons. 18/20 had a positive scan, 3 of these were negative for radiological changes. 2/20 horses had radiographic but not scintigraphic changes. 13/20 horses were positive in all three procedures.

Drug administration

The owners experienced no difficulties with the administration of CaPPS and no adverse reactions were noted.

3. Assessment of outcome

These results will be discussed in the following chapters:

1) Functional outcome	- Lameness scores and questionnaires	- Chapter 5.
2) Measures of anatomical changes in joints	- Radiography	- Chapter 6.
3) Measures of disease process	- Scintigraphy	- Chapter 6.
	- Biochemical markers	- Chapter 9.

Discussion

This is the first clinical study to be carried out using CaPPS in clinical cases of OA in the horse. Although the importance of investigating the efficacy of this drug in clinical cases is paramount clinical trials are difficult to perform and many difficulties did indeed present themselves during the course of this pilot study.

Trial design

1. Reduction in bias

Comparison with a control

This was achieved by comparison of the suggested dose of CaPPS with a low dose which was advised by the manufacturer (unpublished data) to be below a concentration necessary to produce any beneficial effect . It would have been preferable to use a placebo as a control, but as stated earlier, owner interest in this was poor and this factor resulted in a considerable delay to the start of the trial. Once a low dose control group had been introduced, owner compliance was good, even though it was explained to the owners that 50% of the horse would still be receiving a very low instead of a therapeutic dose. The lack of acceptance of the need for the placebo group by the owners was a major drawback, and highlighted the need for education of owners concerning benefits of correctly conducted clinical trials.

Randomisation and blinding

Initially , on the basis of the trial being placebo controlled, and in the hope that 40 horses could be recruited to the trial, it was intended to use a completely random process to assign horses to either drug or placebo, and for this purpose a computer was used to generate a random allocation. Numbered bottles of either 10 mls of CaPPS or placebo were provided by the drug company (Biopharm Australia) along with corresponding numbered sealed

envelopes containing the identity of the bottle contents. However, this plan had to be altered because of the poor owner compliance and because of the ensuing time delay it became apparent that only half of the intended number of horses could be recruited to the trial. By this stage it had also become clear from other studies (Fuller *et al.* 1996) that different joints affected by OA could not be compared in the measurement of marker levels. For these reasons it was decided to use the system of stratified randomisation. Therefore with the help of a person not involved with the trial (MP), dosages of CaPPS in the bottles were reorganised and labelled A or B, the identification of A and B being known only to the independent assistant. This person then arranged an order for allocation of cases, according to the joint in which OA was diagnosed. This enabled, over the course of the trial , the numbers of cases of OA in different joints to be equal in each group.

Blinding was easily achieved in this trial by the use of this independent person (MP) who kept the identity of dose A and dose B blinded until the end of the trial. There was no difference in the appearance of the drug within the bottles, and no reactions to any of the drug administrations were noted.

2. Protocol

Null hypothesis

Whether or not the null hypothesis was rejected will be discussed in following chapters which relate to the results of the trial.

Numbers and duration

Ideally it would have been preferable to extend the course of the trial over a prolonged time period, but this was limited by the period of time available for the study. It would also seem unlikely, for economic reasons, that owners could be persuaded to keep an inactive horse for a lengthy time period. However, the duration of this study was longer than any of the controlled trials reviewed in the literature. It was not possible to extend the numbers suitable for the trial beyond 20 in the available time. The problem with inadequate sample size is a common one in veterinary clinical trials, which is often overcome by carrying out multicentre trials. However, the disadvantage of this is the necessary involvement of several assessors thus increasing variability, - in this case, one assessor (CF) was responsible for every assessment at each time point.

Inclusion criteria

It was surprising how difficult it was to reach an accurate diagnosis of OA in many horses referred to the veterinary hospital with pre-existing diagnoses of OA (For equine OA diagnosis see Chapter 2). This finding indicated that without detailed and thorough diagnostic examinations many horses may be diagnosed as suffering from OA when perhaps the term joint disease may be more appropriate. In man, the gold standard for the diagnosis of OA is still the radiograph (Kellgren *et al.* 1957) and a patient will not be diagnosed as OA until certain documented changes are visible i.e. subchondral bone sclerosis, subchondral bone cysts, narrowing of the joint space, and osteophytes. Atlases providing more detail for various joints are available (Altman *et al.* 1995). Patients suspected of having OA but showing no radiological signs are classified as being pre-radiological. Horses are relatively slow in laying down new bone and thus commonly present showing clinical signs of OA but without radiographic changes until the condition is very advanced. Some human researchers therefore believe that equine cases seen without radiological changes should be classified as pre-radiological and therefore cannot be classified as suffering from OA, while others believe that veterinarians have the advantage of seeing their patients at a much earlier stage of disease. However, without the help of radiographic changes, definitive diagnosis of OA can be problematic. Intra-articular analgesia is helpful in localising the site of pain to the joint, but a positive result does not necessarily mean that OA is the cause of pain, inflammation of the synovium only for example could be responsible. Likewise, a negative result does not necessarily rule out the possibility of subchondral bone pain caused by OA being present.

Scintigraphy has been shown in man (Dieppe *et al.* 1993) to predict subsequent OA change in the human knee joint, although results must be interpreted with care to avoid influence of variables (Devous *et al.* 1984). In this study 3/20 of the horses on the trial had a positive scintigraphic but a negative radiographic result.

Because of the limitations of each diagnostic technique when used alone, it was decided to diagnose OA on the basis of a positive result in two out of the three techniques.

Inclusion criteria in this trial were clearly defined and were clinically relevant. It may have been preferable to state exclusion criteria which would have excluded those horses with a duration of disease already exceeding 8 months at the beginning of the trial. It would also have made the population studied more homogeneous if only certain breeds had been included, but this would not have been practical, and it may be argued that by including various types of horse the results of the trial may be more applicable to the general population.

Instructions and consent

One aspect of the trial which was reflected in the owner assessment of their horses' progress, as will be seen later in the chapter concerning functional outcome, was their individual aspirations for the future of their horse. Some owners hoped only for their horse to continue to be of some use as an occasional riding horse, while others expected that a positive outcome to the trial would mean a return to competition fitness. This, along with their individual personality, undoubtedly had some unavoidable effect on their judgement of the trial's success. Owners were instructed to keep their horses in light exercise, however, some found this impractical and preferred to turn their animal away to rest at grass for the duration of the trial. Some increased the exercise as the trial progressed and their horse improved, and some varied the periods of rest and exercise with the seasons. This undoubtedly would have added another variable to the trial. The difficulty in persuading owners to persist with a particular management regime over a prolonged time period is one reason for the majority of clinical trials being carried out for a short duration only (Gaustad and Larsen 1995).

3. Assessment of outcome

Clinical trials in rheumatology are said to be only as good as their endpoints (Ratain and Hochberg 1990) . The endpoints, or measures of outcome in a trial should be validated (Deyo *et al.* 1991) and appropriate to the drug under investigation and the properties possessed by that drug. One of the aims of this trial therefore was to attempt to validate one of the most frequently used outcome measures in veterinary clinical trials of this type, lameness scoring. The WOMAC scoring scale for measuring pain and physical function (Bellamy *et al.* 1985) has been validated in human studies but to date no validation studies of lameness scoring have been carried out in veterinary medicine. Lameness scoring in horses is a well established technique and is clinically relevant thus making it a suitable method of measuring outcome in OA, but it remains subjective. Without prior knowledge of the reliability of a measure of outcome, the variability in the normal population and the degree of change necessary to indicate a positive improvement, the power of any clinical trial cannot be calculated. The use of a camcorder to record lameness at each examination with subsequent independent blinded lameness scoring allowed the reliability of this method to be assessed, and the results of this are reported in Chapter 5. The use of owner questionnaires to assess equine OA has not been previously reported in the veterinary literature, but in a recent canine OA study a questionnaire has been evaluated and was found to be reliable (Innes 1997).

This trial also provided an opportunity to assess other methods of measuring OA i.e. scintigraphy and biochemical markers, in a clinical situation. More studies need to be carried

out to investigate the normal variation of these parameters before either can be accurately validated. The use of these measures in this trial will be discussed in later chapters.

4) Statistics

Although sample size estimation and power is crucial to the design and planning of a randomised clinical trial, it is not possible to calculate this accurately without prior knowledge or experience of the variance of the outcome measure. It is surprising when reviewing the veterinary literature to find that no study made reference to these calculations. Even when sample size has been accurately calculated, it is often difficult to achieve when depending on the availability of suitable clinical cases which meet the strict inclusion criteria. Low numbers in the trial is one factor which can increase the chance of a Type II error being made (Ratain and Hochberg 1990). It has been stated that in any study where no significant differences are found between treatment groups, calculations of the power should be shown (Markel 1991) but in the literature reviewed, this was not done.

In this study the use of stratification was helpful for the purposes of statistical analysis because horses could then be matched between groups for joint site.

Summary

In summary, a prospective randomised and blinded pilot clinical trial was performed. This provided the opportunity to validate various outcome measures and to make preliminary assessments regarding the efficacy of CaPPS in clinical cases of OA in the horse. This study highlighted the problems involved both in the definitive diagnosis of OA in the horse, and with veterinary clinical trials, but nevertheless the experience of conducting such a trial served to emphasise their importance in veterinary research and the methods by which variability should and can be minimised.

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Chapter Five

The assessment of clinical outcome.

Introduction

In this chapter the clinical assessment of the calcium pentosan polysulphate (CaPPS) clinical trial will be reported. When assessing clinical, or functional outcome, chosen measures should be valid, reliable and responsive (for definitions see later), and ideally they should have been previously published in other studies so that comparisons of results can be made.

In the recommendations for human clinical trials of drugs used to treat osteoarthritis (Altman 1990) it is suggested that a core of validated measures should be included with others being added optionally, i.e.

Validated measures : Pain, physical function, patient global assessment, imaging (for studies over 1 year in duration).
Additional measures : Quality of life, physician global assessment.
Optional measures: Signs of inflammation, biologic markers, stiffness, performance based measures, presence of flares, time to surgery, consumption of analgesics.

For studies designed to affect symptoms the primary outcome variable should usually be pain as reported by the patient. For disease modifying osteoarthritis drugs (DMOADS) the primary outcome variable should be a measure of joint morphology e.g. imaging techniques. In man, the use of radiography, but not yet magnetic resonance imaging (MRI), arthroscopy, or scintigraphy has been validated.

For the purposes of this chapter subjective outcome measures relating to clinical assessment of symptomatic osteoarthritic drugs (SYSADOAS) will be discussed. Imaging as an outcome measure of a DMOAD will be discussed in Chapter 6.

1. Pain

Pain assessment is a subjective but essential part of disease assessment in man. However it is difficult to quantitate, and there is considerable variability, between patients, and throughout the duration of the disease (Bellamy *et al.* 1990). According to the recommendations for the design of clinical trials in OA in man (Altman *et al.* 1996) the degree of pain can be recorded

on a five-point Likert scale i.e. None, Mild, Moderate, Severe and Very severe, or on a 10 centimetre Visual Analogue Scale (VAS). There is considerable debate as to which is the better scale to use.

A validated semi-objective pain questionnaire may also be used e.g. the Western Ontario and McMaster Universities Scale (WOMAC) (Bellamy *et al.* 1985), the Health assessment questionnaire (HAQ) (Fries *et al.* 1980), and the Arthritis impact scale (AIMS) (Meenan *et al.* 1980).

2. Physical function

The function subscale of the WOMAC has been validated (Bellamy *et al.* 1985) and is recommended for use in OA of the hip and knee in man.

3. Physician global assessment

Global assessments are the simplest composite index. Where there is no gold standard for the measurement of disease activity these remain the "next best thing" and are frequently used in the assessment of criterion validity (Symmons 1994). Physician global assessments are more objective than patients assessment of their own pain, but they can still be influenced by personal impressions and emotions. Signal measurements, based on well defined individual measurements, are less subjective than global and are preferred by Bellamy (Bellamy *et al.* 1990). There is much debate as to the best method when assessing arthritis in man - it seems to depend on the individual assessor i.e. whether a "lumper" or a "splitter" (Kirwan 1994).

4. Patient global assessment

This should be measured on a VAS or Likert scale although the optimal method has not been established.

Recording scales

1. Visual analogue scales.

These are probably the most popular scales used in pain assessment. Visual analogue scales (VAS) consist of 10cm line, which can be horizontal or vertical and is limited by endpoints described as severe pain or no pain. VAS scales are assumed to be more sensitive than Likert scales because of the infinite number of points on the scale. However there are inherent sources of error which can limit the accuracy of the results. Some studies have shown a clustering of results at the so called "golden section" of the scale i.e. 68% of the way from each end of the VAS (Dixon *et al.* 1981; Hinchcliffe *et al.* 1985). It has also been reported that the precision of results along the scale can vary, results being more precise near the ends

of the VAS and less so near the golden section (Dixon and Bird 1981). This is of particular importance when the scale is used to assess longitudinal changes in pain.

When using VAS to record serial pain measurements it is debatable whether patients should be allowed access to their previous scores. It has been suggested that patients may either make an overestimation (Huskisson 1976) or minimise (Vandenburg *et al.* 1984) their pain when previous recordings are unavailable. However these studies indicate that access to previous results may cause bias, and therefore since blinding removes bias it seems more appropriate that previous results should not be viewed (Bird *et al.* 1987).

2. Likert scales

These five point descriptive scales were first reported by Likert in 1932 (Likert 1932) and then in rheumatology by Keele in 1948 (Keele 1948) and are still the preferred method of some researchers today. Likert scales used in human trials of RA were found to be capable of consistently detecting statistical differences between active drug response and placebo (Bellamy *et al.* 1984). However other sources (Bird and Dixon 1987) report that descriptive pain scales lack sensitivity and a patient's pain may improve but fail to change from one category to another. These scales are also prone to a floor or ceiling effect (Bindman 1990) and it has been reported that the maximum category possible may not correlate accurately with that indicated by a VAS scale (Welsh *et al.* 1993).

3. Other scales

These include numerical rating scales which are similar to the Likert but use numerical values instead of descriptive terms, and the pain faces scale which shows a range of faces in varying degrees of pain and was designed primarily for use in children. The McGill questionnaire uses a more complex pain index to investigate the sensory, affective and evaluative qualities as well as the degree of pain (Melzack 1975).

Assessment of outcome measures

The characteristics of a good outcome measure include:-

1. Validity.

The validity of a measure is the ability to measure what is intended . There are different categories of validity, i.e.

a) Content validity - the completeness of an index to cover all the important areas that should be measured.

Face validity - one form of content validity, an assessment of whether the index is meaningful and relevant.

b) Criteria validity - the correlation with another validated measure or gold standard.

c) Construct validity - the plausible relation to other measures used.

2. Reliability

The reliability of a measure is the ability to consistently produce the same results.
To measure reliability, intra- and inter- observer variation should be calculated. This is best done by test and retest at one to two week intervals (Deyo *et al.* 1991). The variability of qualitative measurements should be calculated using the Kappa statistic (Fleiss 1981) while for quantitative measurements the Intraclass correlation coefficient (ICC) (Deyo *et al.* 1991) is more suitable to measure the concordance i.e. the strength of the correlation and also whether the slope and intercept vary from replicate measures. The ICC is mathematically equivalent to kappa (nominal data) and weighted kappa (ordinal data).

3. Responsiveness

The responsiveness of an index is the sensitivity to change. Any measure chosen should be able to change and should have a small standard deviation (Symmons 1994). The responsiveness of a measure can be assessed by using it to measure change following an intervention with a known outcome.

Trials in animals

Obviously in any trials performed in veterinary medicine no outcome measure can be purely subjective because response must be measured by owner or physician. Outcome assessed by physician is generally accepted to be more objective than owner assessments which are often affected by emotional or economic involvement. In equine trials the most commonly used outcome measure is an index of physician assessed pain, indicated by lameness (Asheim 1984; Aviad *et al.* 1988; Gaustad *et al.* 1995; Verschooten *et al.* 1997; White *et al.* 1994) and although this is a well established and relevant method, there are no reports of validation of this method in the literature. Another outcome measure commonly used is that of return to athletic performance (Asheim 1984; Phillips 1989; Verschooten and Desmet 1997; White *et al.* 1994) . Efforts have been made to quantify lameness in the horse both by kinematic methods (Buchner *et al.* 1993; Pourcelot *et al.* 1997) and by the use of force plates (Auer *et al.* 1980; Aviad 1988; Gingerich *et al.* 1979) . In one study (Back *et al.* 1993) lameness scoring by one clinician compared favourably with results from kinematic apparatus.

Although questionnaires, or metrology, are a widely used and validated method of acquiring information and measuring change in human trials, no reports of the use of questionnaires in the assessment of such trials in horses have been found in the literature.

VAS scales have been used recently in the assessment of postoperative pain in animals (Lascelles *et al.* 1994) and there are reports of Likert scales being used in the assessment of chronic pain caused by foot rot in sheep (Ley *et al.* 1994). A comparison of a VAS and a numerical rating scale in the assessment of lameness in sheep was made in 1993 (Welsh *et al.* 1993) which concluded that both scales compared favourably with respect to repeatability, reproducibility and use by 2 observers, but the VAS scale was inherently more sensitive.

Patient assessed pain obviously becomes owner assessed pain in veterinary trials and thus the accuracy of these results will depend on the observational skills of the owner. Recently in a study investigating use of questionnaires in the assessment of canine lameness following surgery (Innes 1997) a questionnaire was designed and found to be reliable and responsive, although the relationship between owner and veterinary surgeon assessments was poor.

Aims

1. To assess the reliability of lameness scores as a system of clinical assessment, intra- and inter-observer.

2. To assess the use of owner questionnaires as valid measurement tools.

3. To assess the relationship between owner and physician impression of lameness.

4. To measure the functional outcome in the calcium pentosan polysulphate trial.

Materials

Mepivacaine Hydrochloride - "Intraepicaine", Arnolds Veterinary Products, Shrewsbury
Camcorder- Sanyo VMD-66P video camera recorder.

Methods

1. Physician assessment

Details of diagnostic and inclusion criteria and demographic variables are described in Chapter 4. In this chapter only the assessment of functional outcome will be reported.

As described in Chapter 4 horses recruited to the clinical study were examined in the same way on four occasions at 3 monthly intervals over a 9 month period. Each examination was carried out in the Department of Clinical Science, University of Bristol, by the same investigator (CF) on each occasion. Before any clinical examination began a full history of the disease process was acquired from the owner of each horse. A visual examination of the horse was then followed by palpation of the limbs, making particular note of the range of motion of the joints, the degree of synovial effusion and periarticular thickening of the joints, and the degree of pain on movement (Figure 5-1). The gait of each horse was observed and recorded with a camcorder first at the walk and then at the trot in a straight line on a level concrete surface, both moving away and then returning towards the investigator. Each horse was then trotted on the lunge in a circle, on both reins, first on a soft and then on a hard surface.

OA CASES - DATA SHEET.

VISIT	1	2	3	4
DATE				
SERUM				
SF -L				
SF - R				
SCAN				
X-RAYS				
ARTHRO				
NO OF JTS				

Name: Study No : Hospital No:

Owner: Scan No: X-ray No:

Age: Sex : Breed : Height : Weight :

HISTORY

Presenting complaint :

Duration:

Previous athletic performance:

Diagnostic procedures and results:

Treatments and response:

CLINICAL EXAMINATION.

Joints affected:	Coffin	Pastern	Fetlock	Carpus	
LF					
RF					
Joints affected:	Coffin	Pastern	Fetlock	Hock	Stifle
LH					
RH					

PALPATION

JOINT:			
Effusion:			
Thickening:			
Pain on movement:			
Range:			

Scoring system : 0 = No 1 = Mild 2 = Moderate 3 = Severe

Range - Normal / Less Extension/Less Flexion

DEGREE OF LAMENESS : LF RF LH RH

Walk:

Trot:

Lunge:

FLEXION TESTS

Joint					
Result (+/-)					

NERVE BLOCKS

Nerve block performed : Result:

Figure 5-1: Example of data record sheet used during trial

Lameness was assessed based on observation of head movement, and symmetry of stride and pelvic movement according to the system described by Wyn-Jones (Wyn-Jones 1988). A score from 0 - 10 ranging from sound to non weight bearing lameness was given for each gait on each occasion. At the end of the trial a global score of the overall change in lameness scores was given.

Lameness compared to beginning of trial	Global score
Worse	-1
Same	0
Improvement	1
Sound all gaits	2

Table 5-1: Global scoring system for lameness improvement

2. Owner assessment

Questionnaires

Two questionnaires were designed to provide a measure of change in degree of lameness as viewed by the owner. These were given to each owner on the first and last visit. The questions in each questionnaire were similar but the first was based on a 10 centimetre Visual analogue and the second on a four or five point Likert scale. Owners were instructed in their use before being given the questionnaires. When filling out the second questionnaire they were not reminded of their responses to the first questionnaire (Figure 5-2a and b). One question was repeated in each questionnaire in order to provide a calculation of the reliability of the owner in accurately recalling information.

Six months after finishing the trial, each owner was sent a third questionnaire. Questions in this form were designed to check reproducibility of the system as well as providing a measure of the progress of the horse since finishing the trial (Figure 5-3a and b).

When assessing the results of the questionnaires, the extremes of the VAS scales were measured either from 0 - 10cm, or from -5 to +5 cm, depending on the question. The descriptive terms used in the Likert scales were converted to numerical values, i.e. either from 0 to 3 for the four point scales, or from -2 to +2 for the five point scales.

Before the lameness Name:

How much exercise was he given ?

|_____|

None Constantly in use

Since the lameness

1. How much exercise do you give him now ?

|_____|

Complete rest Exercise as usual

2. If still at work, or at the lunge - does the stiffness wear off with exercise ?

|_____|

Not at all Completely

3. How well can he move around when free i.e. in the stable / field ?

|_____|

Stands still all the time Moves normally

4. How much time does he spend lying down ?

|_____|

All the time Never seen lying down

5. What degree of stiffness / lameness do **you** think he shows ?

a) At the walk

|_____|

Completely lame Completely sound

b) At the trot

|_____|

Completely lame Completely sound

Figure 5-2a: Visual analogue scale questionnaire - outset of trial

Please tick the one response which best describes the situation.

1. Before the problem, how much work was your horse given ?	None	Occasional	Frequent	Regular
	-------	-------	-------	-------
2. Since the onset of lameness, how much work does he do now ?	None	Occasional	Frequent	Regular
	-------	-------	-------	-------
3. Does the stiffness/lameness, wear off with movement ?	No	Slightly	Mostly	Completely
	-------	-------	-------	-------
4. How well does he move in field/stable when free ?	Extreme Difficulty	Much Difficulty	Some Difficulty	No Difficulty
	-------	-------	-------	-------
5. How often is he seen lying down?	All time	Frequently	Occasionally	Never
	-------	-------	-------	-------
6. How lame do **you** think he is ?	Extremely	Very	Slightly	Sound
a) At walk	-------	-------	-------	-------
b) At trot	-------	-------	-------	-------

Figure 5-2b: Likert scale questionnaire - outset of trial

Before the lameness Name:

How much exercise was he given ?

|_____|

None Constantly in use

Since the end of trial

1. How much exercise do you give him now ?

|_____|

Complete rest Exercise as usual

2. If still lame, what degree of stiffness / lameness do **you** think he shows ?

a) At the walk

|_____|

Completely lame Completely sound

b) At the trot

|_____|

Completely lame Completely sound

3. At the end of the trial (compared to the beginning), do you think he was

|_____|_____|

Much worse Same Much better

4. How do you think he is now (compared to the end of the trial)

|_____|_____|

Much worse Same Much better

5. How successful do you think the CaPPS treatment was ?

|_____|

No use Excellent

Figure 5-3a: Visual analogue scale questionnaire issued 6 months following the end of the trial.

Please tick the one response which best describes the situation.

1. Before the problem, how much work was your horse given ?	None	Occasional	Frequent	Regular
	------	------	------	------
2. Since the end of the trial, how much work does he do now ?	None	Occasional	Frequent	Regular
	------	------	------	------
3. How lame do you think he is now?	Extremely	Very	Slightly	Sound
a) At walk	-------	-------	-------	-------
b) At trot	-------	-------	-------	-------

4. At the end of the trial (<u>compared to the beginning</u>),do you think he was	Much worse	Slightly worse	Same	Slightly better	Much better
	------	------	------	------	------
5. How do you think he is now (<u>compared to the end of the trial</u>)	Much worse	Slightly worse	Same	Slightly better	Much better
	------	------	------	------	------
6. How successful do you think the CaPPS treatment was ?	Useless	Poor	Moderate	Good	Excellent
	------	------	------	------	------

Figure 5-3b: Likert scale questionnaire issued 6 months following end of the trial.

Name of horse : **Date :**

1. How long ago did you finish the trial ?

2. Do you still own the horse ?

3. If not, do you know how he is now ?

4. If he is still lame, how lame, and on which leg ?

5. If he is sound, for what purpose are you now using him ?

6. Have you used any further treatment for his OA since the trial ?

7. If so which one (CaPPS, Adequan, Hyonate for example) ?

8. What was the effect of this treatment ?

9. Please add any other information relating to the lameness that you feel may be relevant.....

Figure 5-4: Information request included with questionnaires issued six months following the end of the trial.

Validation of outcome measures

The chosen gold standard for improvement in functional outcome was the global scoring derived from the video recording of lameness. Other parameters were compared against these scores in order to test their criterion validity.

Interobserver variation of lameness scoring.

At the end of the trial the camcorder recordings of the gaits of the horses were copied onto two videotapes. On one videotape (V1) the recordings for each horse at each examination were kept together , in order, but the allocation of each horse to a particular treatment group was kept blinded. This videotape was then viewed, in single sittings, by three independent veterinary clinicians and a global score of improvement during the trial (Table 5-1) was given to each horse.

Intra-observer variation of lameness scoring

To measure intraobserver variation, one clinician (CF) scored the same videotape (V1) on two occasions, 14 days apart.

Reliability of lameness scoring at the time of examination

The same investigator who scored all the horses for lameness at the time of examination (CF) viewed a second videotape (V2) on which the recordings of lameness were blinded to date order for each horse. The agreement between the results was calculated using the kappa statistic.

Statistics

Comparisons between outcomes were made using the paired students t test where results were normally distributed and the Wilcoxon signed rank test where results where skewed.
The difference in global score of outcome between the high and low dose groups was compared using Fishers exact test.

Intra and inter-observer variation of global lameness scores was measured using the Kappa statistic (κ) (Fleiss 1981) and was calculated using Microsoft Excel on a Macintosh computer. Weighted kappa values should be calculated for ordinal variables whereas if variables are nominal unweighted kappas should be used. If disagreements between ordinal variables are rarely more than one category, it is acceptable to use unweighted kappas. A kappa value of below 0.4 was considered unacceptable variability, whereas a value of over 0.75 was considered excellent .

Reliability of owners in using questionnaires was assessed by using the Intraclass correlation coefficient (Deyo *et al.* 1991) to measure the concordance between responses to repeated questions on questionnaires with quantitative values i.e. the VAS. Kappa values were calculated to assess the reliability of questions with ordinal values i.e. Likert scales.
Correlation between the various parameters assessed by the questionnaires was calculated using the nonparametric Spearman correlation coefficient and confidence intervals were stated at 95% and significance at 5%. For these correlations the statistics software Instat was used.
For power and sample size estimations see Chapter 4.

Results

1. Physician assessment

Lameness scores as measure of functional outcome

There was no significant difference between the high and low dose groups in lameness scores at the outset of the trial. Lameness scores were then analysed cross sectionally at the 4th examination, and there were still no significant differences in lameness scores (Students t-test) between groups (Table 5-2).

	Low dose group n = 9	High dose group n = 9	Both groups n=18
Outset of trial	2.33 (0.87)	2.06 (1.13)	2.19 (0.98)
Trial conclusion	1.00 (1.22)	1.44 (0.88)	1.22 (1.06)
Significance	0.04	0.06	0.006

Table 5-2: Mean (Standard deviation) lameness scores at beginning and end of the trial for each treatment group and overall change.

7/9 horses in the low dose group and 6/10 horses (Table 5-3) (Horse 6 not shown in Table 5-2 since it did not complete the trial but the global score to 3rd exam = 2) in the high dose group improved to some extent during 9 months of the trial. There was no significant difference (Fishers exact test) between the improvement in horses in either group as measured by global scores (Figure 5-6).

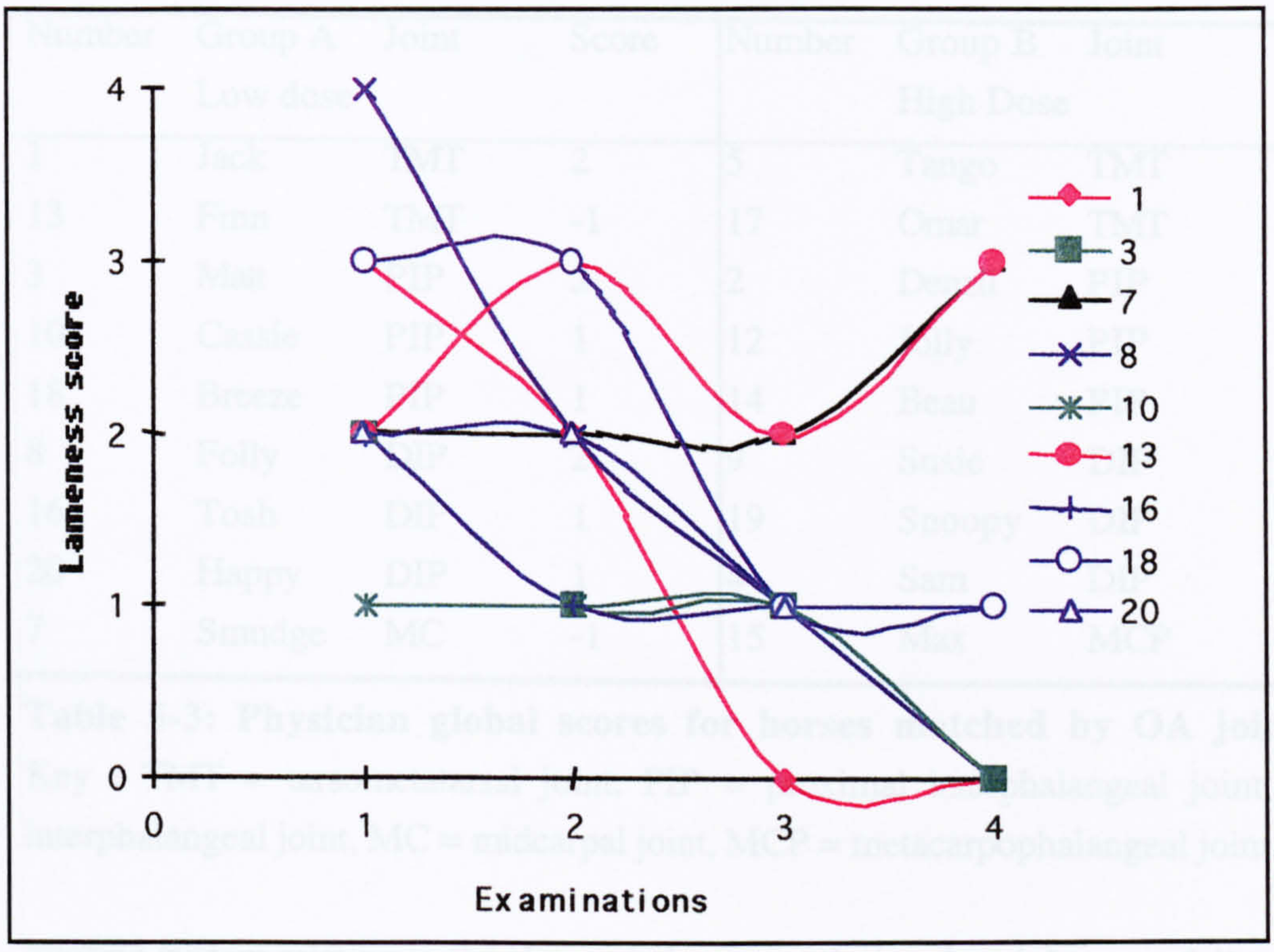

Figure 5-5a: Longitudinal changes in lameness in low dose group

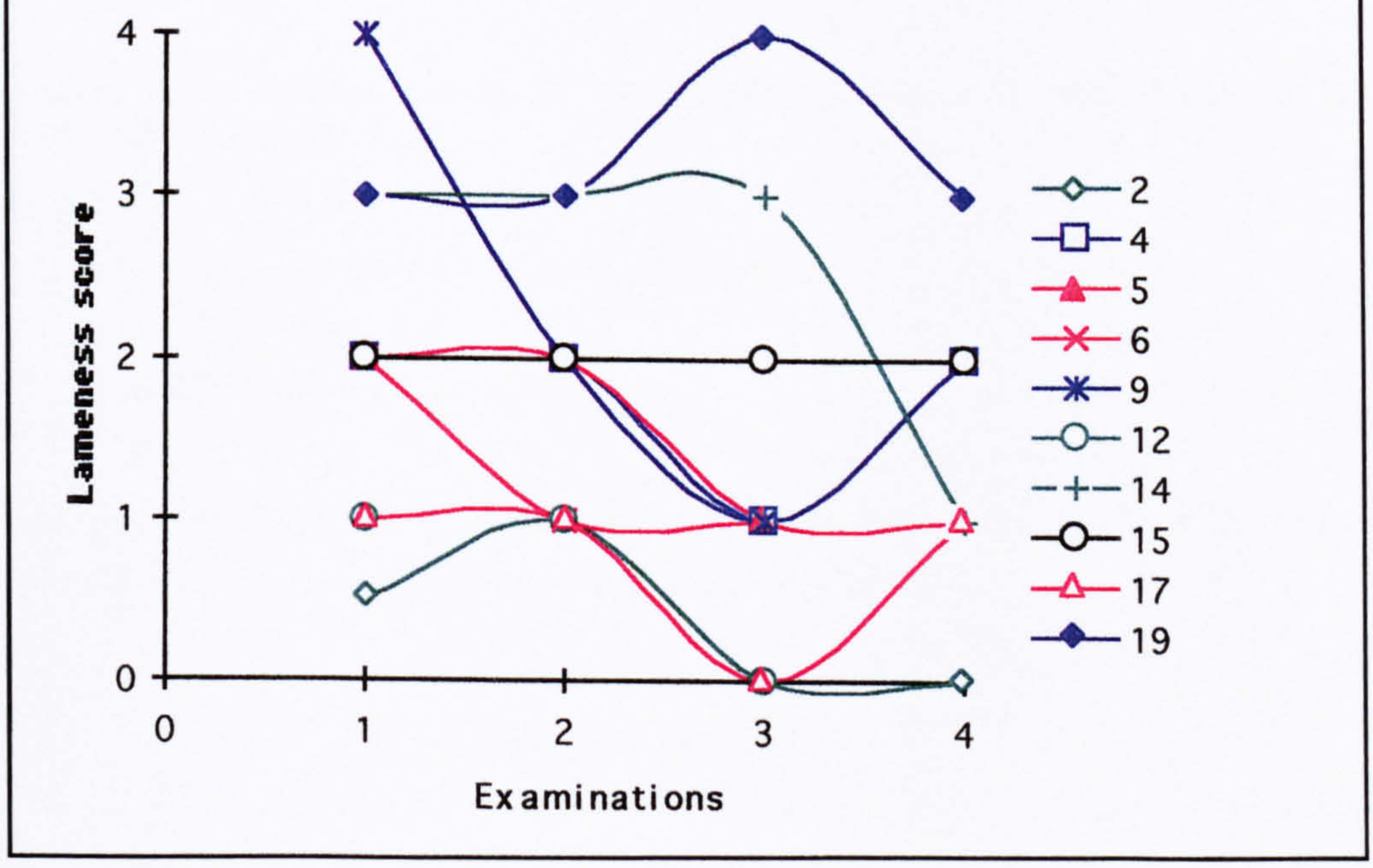

Figure 5-5b: Longitudinal changes in lameness in high dose group

Number	Group A Low dose	Joint	Score	Number	Group B High Dose	Joint	Score
1	Jack	TMT	2	5	Tango	TMT	1
13	Finn	TMT	-1	17	Omar	TMT	1
3	Matt	PIP	2	2	Denzil	PIP	1
10	Cassie	PIP	1	12	Jolly	PIP	0
18	Breeze	PIP	1	14	Beau	PIP	1
8	Folly	DIP	2	9	Susie	DIP	1
16	Tosh	DIP	1	19	Snoopy	DIP	-1
20	Happy	DIP	1	4	Sam	DIP	0
7	Smudge	MC	-1	15	Max	MCP	0

Table 5-3: Physician global scores for horses matched by OA joint.

Key : TMT = tarsometatarsal joint, PIP = proximal interphalangeal joint, DIP = distal interphalangeal joint, MC = midcarpal joint, MCP = metacarpophalangeal joint.

Scoring system relating to the change in lameness during the trial was as follows

-1 = Worse, 0 = Same, 1 = Improved, 2 = Became sound

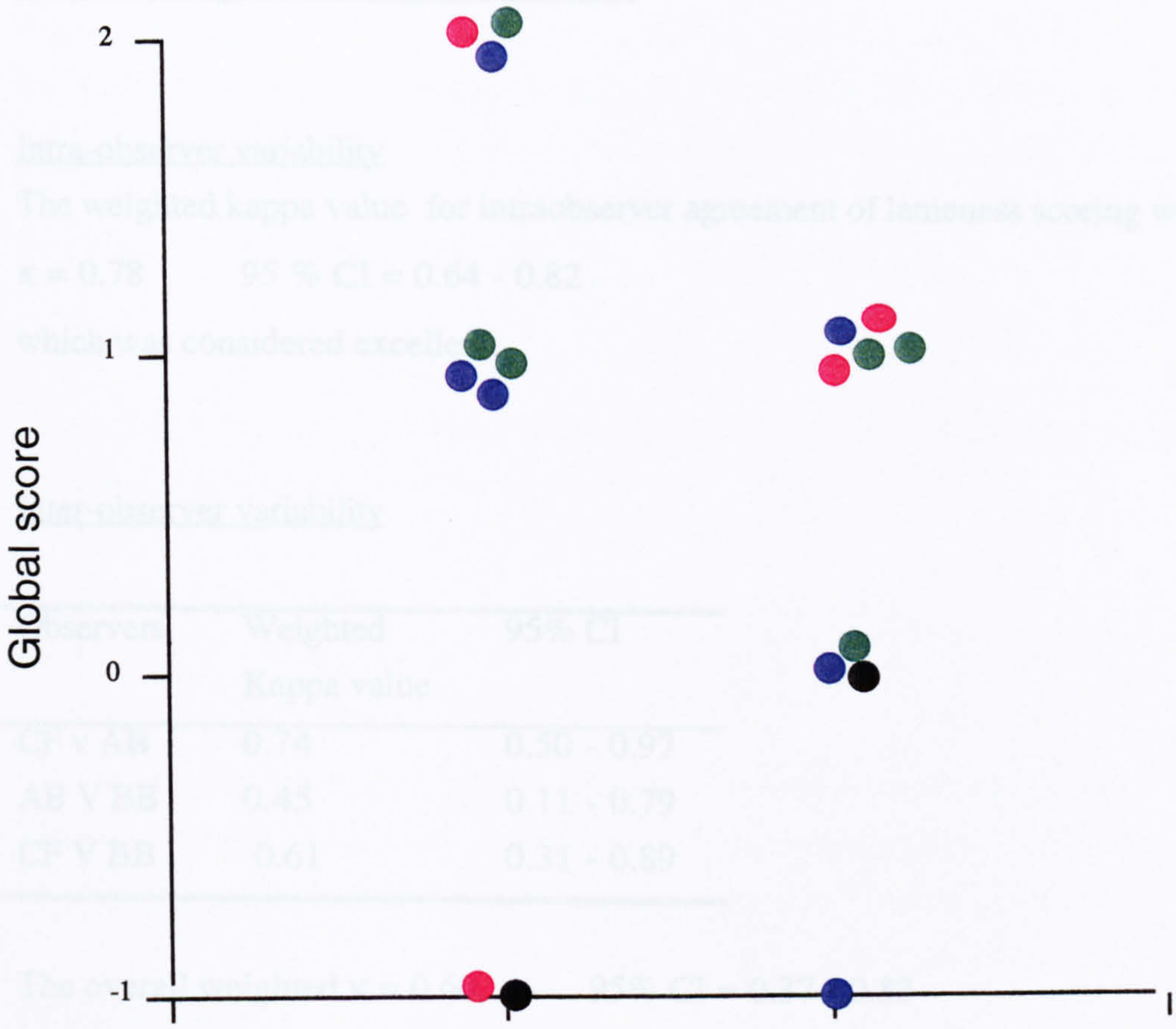

Figure 5-6: Global scores of improvement for matched joints in low (A) and high (B) dose groups

Key : TMT = Red

PIP = Green

DIP = Blue

MCP and MC = Black

Scoring system relating to the change in lameness during the trial was as follows

-1 = Worse, 0 = Same, 1 = Improved, 2 = Became sound

Reliability of gold standard video scores :

Intra-observer variability

The weighted kappa value for intraobserver agreement of lameness scoring was

$\kappa = 0.78$ 95 % CI = 0.64 - 0.82

which was considered excellent.

Inter-observer variability

Observers	Weighted Kappa value	95% CI
CF v AB	0.74	0.50 - 0.97
AB V BB	0.45	0.11 - 0.79
CF V BB	0.61	0.31 - 0.89

The overall weighted $\kappa = 0.60$, 95% CI = 0.37 - 0.83

This was also considered very good agreement. The system of global scoring was judged therefore to be a reliable method of measuring improvement in lameness.

Criterion validity of global scoring of lameness

This could not be measured directly since there was no validated gold standard with which to compare this method. However, since the global scoring of improvement was found to be reliable from scoring blinded video recordings, it was chosen as the "gold standard" within this study with which to compare other methods.

The variation of the global score derived from the lameness scores given at the time of examination with the global score assigned from the video recordings of lameness for one observer (CF) was

$\kappa = 0.58$ (unweighted) 95% CI = 0.51 - 0.65

There was a very significant positive correlation between change in lameness score given at the time of examination (change in lameness between examination 1 and 4) with the global score given from the video of lameness (Spearmans correlation coefficient r_s).

$r_s = 0.79$ 95% CI = 0.51-0.92 P = 0.0001

The variation between lameness scores given at the time of examination with those from the time blinded video for one observer (CF) was

$\kappa = 0.52$ (unweighted) 95% CI = 0.48 - 0.63

The above figures imply good agreement between lameness scoring at the time of examination with subsequent scoring from blinded videos, which indicates that lameness can be consistently and reliably graded by one observer at 3 month intervals.

Responsiveness

This could not be measured directly without the opportunity to test the global lameness scoring by using an intervention of known outcome. However, in this case, from clinical experience it would be acceptable to assume that 9 months would be sufficient to detect a difference using lameness scoring as the outcome variable.

2. Owner assessment

Questionnaires

Two horses did not complete the trial (6 and 11) and therefore only the first questionnaire at 0 months was completed. One owner (9) failed to complete either questionnaire despite being sent reminders. One question (No 3) was disregarded from the questionnaire since 9 owners felt it was not applicable.

As measures of outcome

No significant differences in outcome between the two treatment groups were found as measured by VAS or Likert scales for any signal or global measurement although both scales detected a significant mean overall improvement in lameness during the trial.

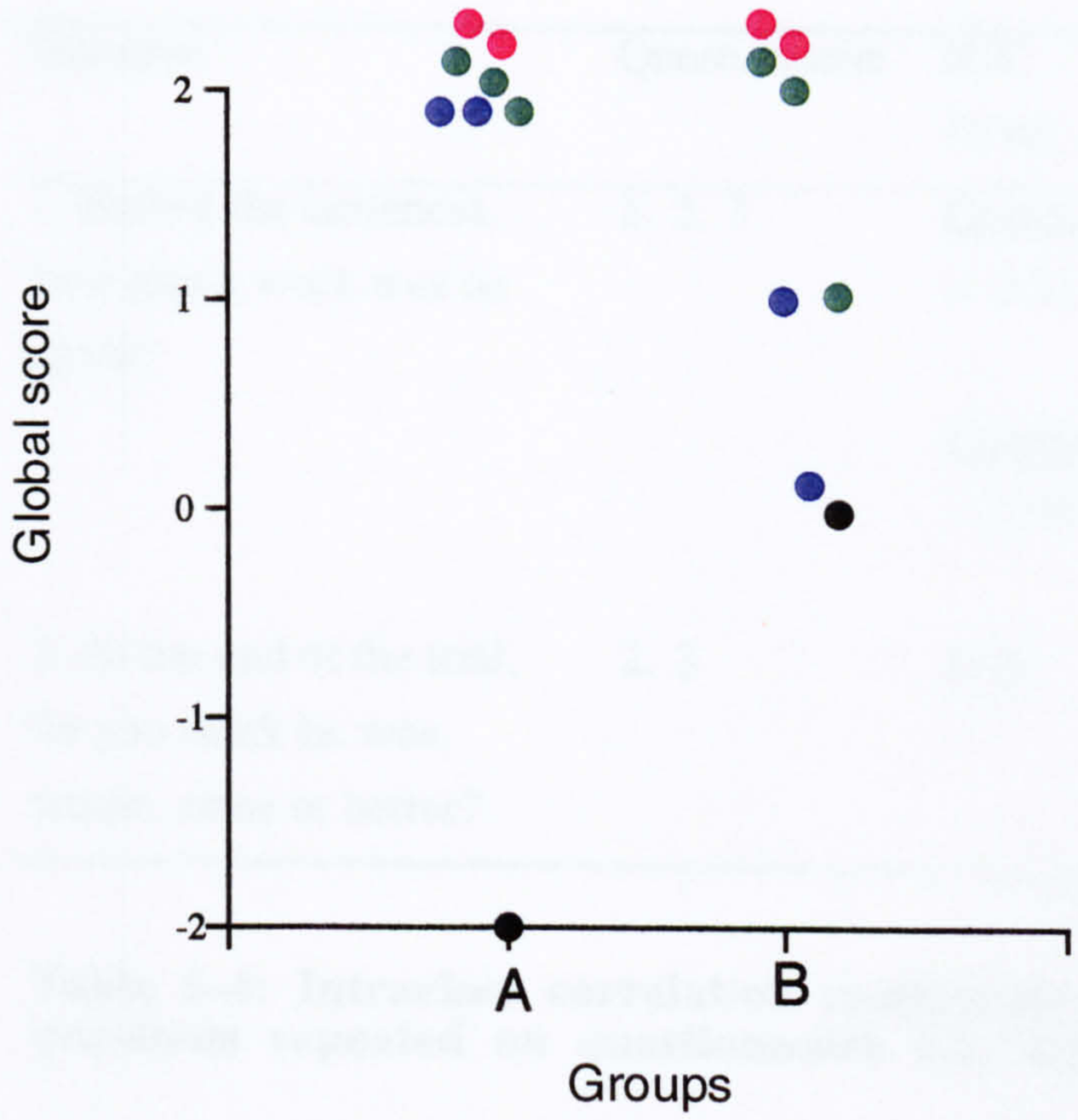

Figure 5-7: Owner global score of horse improvement during trial (Likert scale)

TMT = Red

PIP = Green

DIP = Blue

MCP and MC = Black

Key Scale -2=Much worse, -1= Slightly worse, 0 = Same, 1 = Slightly better, 2= Much better

Reliability of questionnaire

The Intraclass correlation coefficients (ICC) for question 1 on the questionnaire 1, 2 and 3, and for question 6 on questionnaire 2 and 3 are given in Table 5-4.

Question	Questionnaire	ICC (VAS)	Weighted Kappa (Likert)
1. Before the lameness, how much work was he given?	1, 2, 3	Comparing 1-2 = 0.82 Comparing 2-3 = 0.94	Comparing 1-2 = 0.44 Comparing 2-3 = 0.50
2. At the end of the trial, do you think he was, worse, same or better?	2, 3	0.88	0.61

Table 5-4: Intraclass correlation coefficients and weighted kappa values for questions repeated on questionnaire 1,2, and 3.

Owners were found to be reliable when answering questions at time intervals of 6 months. The weighted kappa for ordinal values should be mathematically equivalent to the ICC for quantitative values. Although there was good correlation between the two scales for the replies given to these questions (r_s ranging from 0.63 - 0.93) it was deduced from these results that owners were more reliable when using a VAS than a Likert scale.

Question		Baseline		Outcome		Change	
		VAS	Likert	VAS	Likert	VAS	Likert
1. How well does your horse move around when free?	R value	0.0039	-0.11	0.27	0.45	0.2	**0.57**
	95% CI	-0.48 - 0.49	-0.57 - 0.41	0.24 - 0.68	-0.05 - 0.77	-0.3 - 0.63	**0.11 - 0.83**
	P value	0.99	0.69	0.28	0.07	0.44	**0.02**
2. How much time does your horse spend lying down?	R value	-0.13	0.45	**0.53**	**0.6**	0.46	0.17
	95% CI	-0.58 - 0.38	-0.05 - 0.77	**0.05 - 0.8**	**0.16 - 0.84**	0.05 - 0.77	-0.35 - 0.61
	P value	0.62	0.07	**0.03**	**0.01**	0.06	0.5
3. What degree of lameness do you think your horse shows at the walk?	R value	-0.33	-0.38	0.39	**0.49**	0.46	**0.61**
	95% CI	-0.7 - 0.19	-0.73 - 0.14	-0.13 - 0.74	**0.001 - 0.79**	-0.04 - 0.78	**0.17 - 0.84**
	P value	0.2	0.14	0.12	**0.04**	0.06	**0.01**
4. What degree of lameness do you think your horse shows at the trot?	R value	-0.09	-0.18	0.46	**0.59**	0.39	**0.56**
	95% CI	-0.56 - 0.42	-0.62 - 0.34	-0.4 - 0.78	**0.14 - 0.84**	0.12 - 0.74	**0.12 - 0.74**
	P value	0.7	0.49	0.06	**0.01**	0.12	**0.02**

Table 5-5: Correlation (Spearmans) between individual questions (signal measurements) and physicians global score of outcome (gold standard)

Statistically significant correlations are highlighted in bold

Criterion validity of questionnaire

From Table 5-5 it can be seen that there was very poor correlation between the VAS recorded signal measurements and the physicians global outcome score. However the correlations between the Likert measurements and the physicians global score for outcome and change in lameness at the walk and trot and for change in how well the horse moved when free were significant.

Correlation of individual changes (Signal) v owner's global scores

There was no significant correlation between the owners opinion of change in lameness during the trial and the owners own global score of improvement during the trial when using the VAS scale, but when using the Likert scale the correlation was significant:

$r_s = 0.73$ 95% CI = 0.36 - 0.89 p = 0.001 (Likert)

Correlations since end of trial

There was a significant correlation between the owners global score of improvement since ending the trial and the owners perceived change in lameness since the end of the trial.

$r_s = 0.62$ 95% CI = 0.17 - 0.86 p = 0.01 (VAS)

There was also a very strong correlation between the owners overall impression of the trial once the dosage had been unblinded (recorded on 3rd questionnaire 6 months after the trial) and their global score of improvement given at the end of the trial.

$r_s = 0.7$ 95% CI = 0.34 - 0.9 p = 0.001 (VAS)

Responsiveness of scoring methods

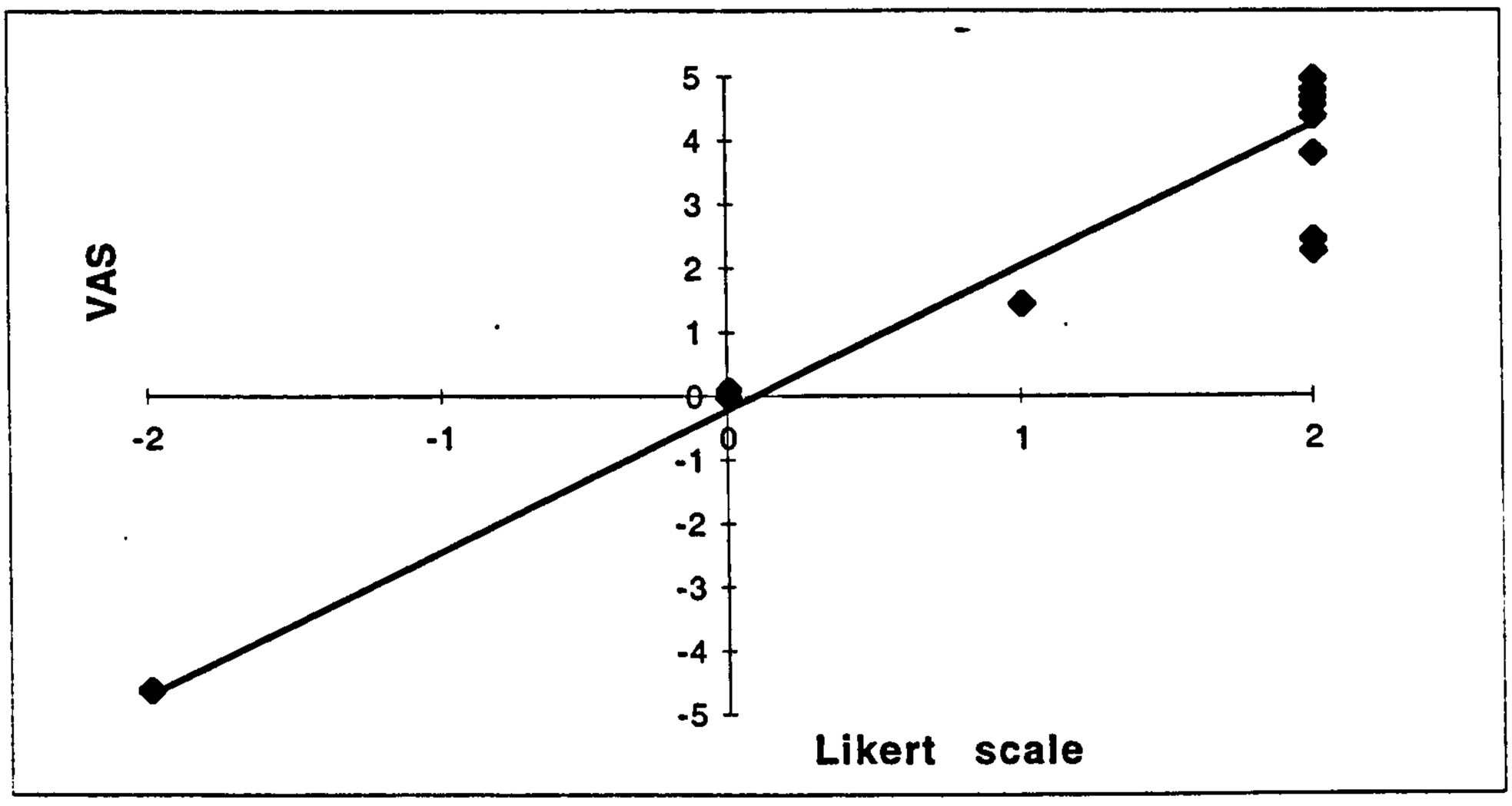

Figure 5-8a: Comparison of Likert and VAS for owner assessed global score

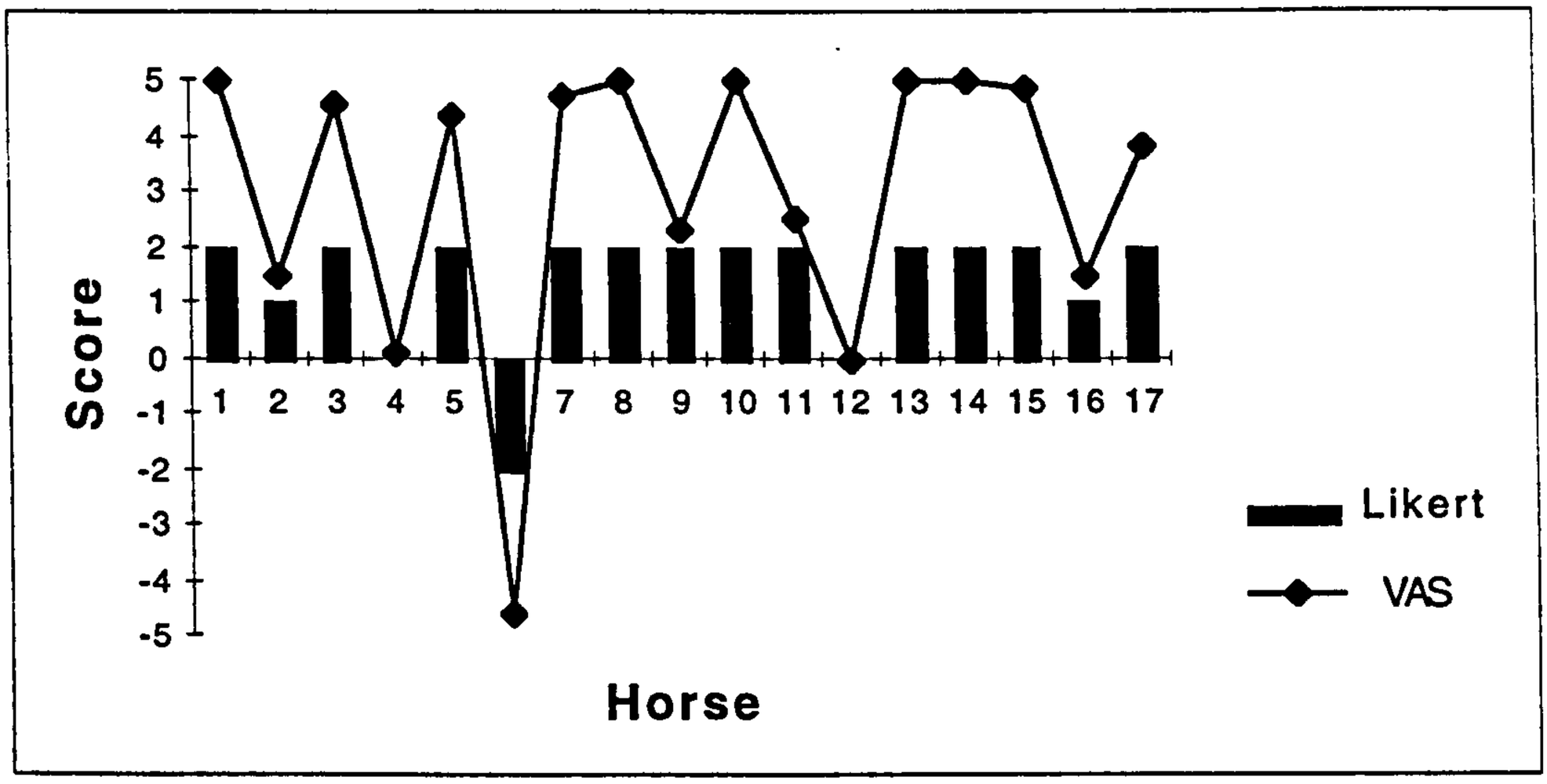

Figure 5-8b: Comparison of Likert and VAS scores for owner assessed global score of change.

Correlation between the two scoring methods was good (Figure 5-8a), i.e. for owner assessed global change:

r_s = 0.78, 95% CI = 0.33-0.93

The VAS scale appeared to be more sensitive cross-sectionally than the Likert which was prone to a "ceiling" effect (Figure 5-8a and b). However, an indication of the responsiveness of the two scoring methods was obtained by comparing the standardised response mean (SRM) on the scores for change in lameness at the trot during the duration of the trial.

	Likert scale	VAS
Mean change	1.29	2.74
Standard deviation (SD)	1.36	3.64
SRM (Mean/SD)	0.95	0.75
Significance of change Paired Students t test	0.004	0.007

Table 5-6: Comparison of SRM and significance of change for two methods of scoring

From table 5-6 it can be seen that although both methods had a high degree of sensitivity to change the Likert scale was actually slightly more sensitive.

The use of the global measurement in the questionnaire in this study were found to have criterion validity when compared to the chosen global standard i.e. physician global score of improvement. However , unlike the VAS, the use of the Likert scale proved to be valid for comparison of the signal measurements also, both with the owners global score and with the physician's global score. The Likert scale was also shown to be more responsive despite seeming less reliable than the VAS.

3. Owner v physician assessment

The correlation between the owners global score, and the physicians global score of improvement as measured from the video recordings, was significant using both scales.

VAS - r_s = 0.51, 95% CI = 0.02 -0.80, p = 0.04

Likert -r_s = 0.59, 95% CI = 0.15 - 0.84 p = 0.01

However the correlations (Likert scale) between the owners global score and the physicians score deduced from the lameness scores at the time of examination were slightly stronger:

Likert - r_s = 0.65 95% CI = 0.23 - 0.86 p=0.005

This implies that the owner may have had an effect on the physician's impression of improvement.

The correlation between the owner's overall impression of the trial and the physicians global score of improvement was only just significant for the Likert recorded measurements however.

r_s = 0.5 95% CI = 0.01-0.79 p = 0.04

4. Questionnaire defined outcome

The results from the questionnaires did not demonstrate any difference in outcome between the treatment groups but they did provide information relating to the future of horses following the trial.

At the end of the trial, out of the 13 owners who answered the question on overall improvement, 11/13 thought their horses were better compared to the beginning of the trial (5/6 B group and 6/7 A group) (Table 5-7).

Horse	Group	Owners global score at end of trial		Owners global score given 6ms after trial		Owners score for change since trial		Overall impression of trial		Sound 6ms later?
		VAS	Likert	VAS	Likert	VAS	Likert	VAS	Likert	
1	A	*	*	5	2	-4.4	-2	4.1	2	no
2	B	*	*	1.5	1	1.4	0	0	0	yes
3	A	*	*	4.6	2	4.2	2	3.7	2	yes
4	B	*	*	0.1	0	1.9	1	0	0	(yes)#
5	B	4.4	2	4.9	2	4.9	2	5	2	yes
7	A	-4.6	-2	-2.5	-1	3.7	2	-5	-2	no
8	A	4.7	2	5	2	5	2	5	2	yes
10	A	5	2	5	2	0	0	4.8	1	yes
12	B	2.3	2	3.1	1	1.8	1	0	0	yes
13	A	5	2	5	2	5	2	5	2	yes
14	B	2.5	2	-0.7	-1	-1	-1	-5	-2	no
15	B	0	0	0	-1	0	0	-5	-1	no
16	A	5	2	*	*	*	*	*	*	*
17	B	5	2	2.4	1	5	2	4.6	1	yes
18	A	4.85	2	4.8	2	0	1	4.8	2	(yes)$^{\alpha}$
19	B	1.5	1	0.9	1	0	0	0	0	no
20	A	3.8	2	4	2	0	0	5	2	yes

Table 5-7: Owner impression of improvement in horse physical function as determined by owner completion of VAS and Likert scales

KEY : * = No reply given on questionnaire

= This horse remained sound as long as maintained in light exercise

α = This horse had received one more course of low dose treatment

VAS scale : -5 to +5 = Much worse/Useless to Much better/Excellent

Likert scale: Much worse/Useless = -2, Slightly worse/Poor = -1, Same/Moderate = 0
Slightly better/Good = 1, Much better/Excellent = 2

Six months after end of trial 11/16 horses were sound (as assessed by owners). 6/8 of these were from the low dose (A) and 5/8 from the high dose group. 9/16 owners thought CaPPS very useful, 7/8 of these being in group A and 2/8 in group B. One owner from group A (7) and 2 owners from group B thought CaPPS was no use (14,15) and 4 (12, 4, 19 and 2) from group B thought it may have helped. Three owners in B group assessed their horses as being sound but remained sceptical about the value of the treatment (2, 4, and 12) while one owner in A group thought the CaPPS treatment good despite her horse still being lame (1).

Discussion

This study has shown that the clinical assessment of lameness is a reliable method both intra- and inter-observer when used as a global score of longitudinal change. The agreement between the lameness scores given at the time of examination with those given from the video recording where lameness recordings were blinded to time was also good indicating that at least intra-observer assessment of individual cross sectional lameness scores is reliable. The good correlation between the global scores deduced from the lameness scores given at the time of the examinations and those from the videotape indicates that it is possible for one observer to score lameness changes reliably in horses presented at 3 month intervals. Since clinician scoring of lameness is the most common method of assessing limb pain and disability in the horse, this is an extremely important finding which is previously unreported in equine medicine.

It was decided to use questionnaires in the trial as a method of providing information relating to the owners own assessment of their animal's progress, and to investigate the validity of the use of owner questionnaires as measures of outcome in equine clinical trials. Owners were found to be reliable in completing the questionnaires, even with intervals of 6 months instead of the recommended 14 days for retesting procedures, and both the VAS and Likert scales were found to be sensitive to change during the trial period. When assessing criterion validity, the Likert scale was found to be superior for signal and global measurements . The owners signal measurements also showed better correlation with the owners overall global score of improvement when the Likert scale was used. The correlation between owner's score of lameness at the end of the trial did correlate with the global score indicating that it was the resulting degree of lameness rather than the change in lameness that influenced their

global score. There was also a good correlation between the physician and owners perception of lameness change. Correlation between owner and physician global assessment of improvement was good indicating that owner questionnaires can be a reliable method of assessing outcome.

In this clinical trial no significant difference in outcome was found between the high and low dose treatment groups using either physician assessment of lameness or owner questionnaires and therefore this trial did not demonstrate that CaPPS is successful as a SYSADOA. Obviously the reason for this lack of difference could be because this was the true result, i.e. accepting the null hypothesis, or alternatively it could indicate a Type II error, i.e. failure to reject the null hypothesis when it is untrue. It has already been discussed (Chapter 4) that the power and sample size of this trial was too small for it to be accepted as anything but a pilot study, and this could have been the reason for the lack of difference detected.

As already stated it was not possible to use a placebo treated group as a control rather than carrying out a dose related study, and in this case any potential effect of a low dose of CaPPS must be further investigated. It is interesting to note that one owner (18) gave her horse another course of CaPPS following the trial at a low dose and judged the effect to be successful.

Another cause of possible Type II error could have been the choice and validity of the outcome measure. The use of lameness scoring as a measure of the degree of pain and impairment of physical function are traditionally used in equine medicine and is a clinically relevant measurement thus indicating face validity to the system. As previously stated, when used as a global score of longitudinal change, lameness scoring was found to be reliable. In this study time did not allow for the inter-observer agreement of cross-sectional lameness scores to be calculated from the time blinded videotape. To do this would be a useful indication of the reliability of cross sectional comparison of severity of lameness which would be applicable to multi-centre and therefore possibly also multi-rater clinical trials. However although cross sectional lameness scores may vary between raters it would still be possible for the overall global score of improvement during the trial to be in agreement which was judged to be the more important measurement in this case.

The reason for the poor correlation between some of the signal scores on the questionnaires could have been because of the choice or the form of those questions. The design of the question was derived from one used in human studies where questions are related to the

ability to perform physical tasks. In horses the main indication of pain or physical disability is lameness which was well observed by the owners . The lack of correlation of the other questions i.e. "how much time is spent lying down" and "how well does he move when free", to the gold standard, may have been because these questions were not clinically relevant, but also could have been because they were less well observed by the owners.

Despite the fact that there was good correlation between owner and physician global assessment of improvement, correlation between the owner impression of the trial overall, however, and the physician assessment of lameness improvement only just reached significance. Correlation does not necessarily equal agreement however and in two cases in particular (13 and 14) the physician and owner assessments were in direct opposition. The owner's overall impression of improvement and the success of the trial takes into account the performance of the horse over a longer time period while the physician's assessment is dependent on one examination every 3 months, so it could be argued that the owner's assessment is therefore more relevant. It would have been interesting to ask the owners to view the videotape recordings of lameness and then to re-evaluate their opinion. It was noticeable that the owner's opinion of the trial success was influenced greatly by their future intention for their horse, some owners expecting their horses to become sound before rating the trial a success, while others being satisfied with any degree of improvement. For this reason it seemed that the physician's more detached assessment of change was likely to be more accurate to use as a gold standard.

It was possible to evaluate the reliability of owners in answering questions over time, by comparing the responses to two questions which were repeated on the questionnaires. It would have been preferable however to have requested that a sample of the owners complete every question on two identical questionnaires at an interval of 14 days, thus enabling the reliability of the questionnaire itself to be assessed.

In this trial the Likert scale showed better criterion validity than the VAS, although both scales were found to be reliable and sensitive to change. When assessing reliability of the scales the ICC was used to measure the agreement in answers on the VAS scale while the weighted kappa was used to assess variability in Likert responses. Although the ICC and weighted kappa are supposed to be mathematically equivalent, the kappa statistic is influenced by very uneven marginal distributions. For example in question 1, most of the Likert scores were 3 with very few scores of 0 or 1. A disagreement in the scores at the low end of the

scale would tend to have a big negative effect on the kappa value which may have been the reason for the discrepancy between the ICC and kappa results.

Despite there being no difference in outcome between the two treatment groups 13/17 owners scored their horses as having improved during the trial, and 11/16 owners thought the CaPPS treatment was useful even though 6 of these owners had horses in the low treatment group. The physician global assessment judged 12/18 horses to have improved clinically during the trial. Many of these owners had tried alternative treatments e.g. intra-articular hyaluronan and polysulphated glycosaminoglycans before the trial without success and only one of these owners felt it necessary to repeat the CaPPS treatment during the 6 months following the trial, and this was done at the low dose. The potential effect of the low dose of CaPPS, possibly as a periarticular anticoagulant, should be investigated further, although there are no reports of efficacy at this dose in the literature. It is possible that the controlled exercise regime instigated during the trial was sufficient to produce a clinical improvement in these horses during the 9 months of the trial and it must be remembered that the traditional treatment of restricting exercise in lame horses may have produced a clinical improvement in this percentage of cases without any other intervention.

Summary

1. Scoring of lameness was found to be a valid and reliable measurement of outcome.

2. The owner questionnaire using the Likert scale was also found to be a valid measurement tool. Owners were found to be reliable in providing information over time .

3. There was good correlation between physician and owner global assessment of lameness improvement.

4. There was no significant difference in outcome between the high and low dose treatment groups in the CaPPS trial. This may have been due to the low sample size and power of the study.

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Chapter Six

Imaging methods for assessing disease outcome

Introduction

In this chapter two methods of measuring outcome by imaging the osteoarthritic joint, which were used in the calcium pentosan polysulphate clinical trial (Chapter 4) will be discussed. Radiography, measuring structural changes in the joint, has long been accepted as the gold standard for diagnosis of osteoarthritis (OA), while scintigraphy, a relatively new technique, provides a measurement of joint physiology and remains to be validated as a reliable outcome measure in clinical trials in OA.

1. Radiography

The Kellgren and Lawrence radiographic grading scheme (K&L) for OA (Table 6-1) (Kellgren *et al.* 1957) was developed in 1957 and was accepted by the World Health Organisation in 1961 as the gold standard for both cross-sectional (Cooper *et al.* 1992; Spector *et al.* 1993) and longitudinal studies (Spector *et al.* 1992) in OA. Despite the fact that radiography has the limitation that it can only be used in diagnosis at a relatively late stage of disease, it has long been considered the primary method of diagnosis in OA. Its accuracy in measuring progression in OA however has only been investigated more recently (Ravaud *et al.* 1996).

The radiographic signs of OA in the horse and in man include narrowing of the joint space, osteophytosis, enthesiopathy, subchondral bone changes, synovial effusion, and thickening of the joint capsule (Cobby *et al.* 1991; Widmer *et al.* 1994). However, not all of these signs are necessarily present in OA, and there is no particular order in which they may occur.

Radiographic features of OA in the horse

Joint space narrowing : The joint space is essentially a measure of cartilage thickness and can therefore potentially be used to assess cartilage loss. The size of the joint space varies in the normal horse depending on the joint imaged (Widmer and Blevins 1994). Proximal, distal and tarsometatarsal joint spaces are usually very thin, while the distal interphalangeal joint space is wide, and the metacarpophalangeal joint is narrower than the interphalangeal joint spaces. In young horses the joint spaces tend to be wider, and marked changes can occur with weight bearing. The exact orientation of the x-ray beam can also produce artificial narrowing of the joint space.

Osteophytosis : Osteophytes are bone deposits within the joint margins caused by secondary osteoarthritic change. Although osteophytes are a reliable radiographic sign of OA, OA may present without osteophytes and they are not indicative of severity or stage of cartilage loss.

Subchondral bone changes : Subchondral bone sclerosis is commonly observed in OA joints but the pathophysiology remains unclear. Identification can be problematic, requiring excellent quality radiographs and sound knowledge of normal variations. Changes may occur at different stages of OA, depending on the joint involved, being common in the proximal phalanx in early metacarpophalangeal OA, and in the third and central tarsal bones in the late stages of OA. Unlike man, subchondral bone cysts are rare in OA in horses. Subchondral bone erosions that do occur are most common in the carpal and tarsal joints.

The features described are used both in the initial diagnosis of OA and also in the quantitative analysis of change in OA. In the horse there is no well defined system described in the literature although various schemes have been used in different studies. In human studies the K&L scheme is still the mostly widely used in OA assessment, but there are doubts about its suitability and reliability.

Grade		Features
0	Normal	No features
1	Doubtful	Minute osteophytes of doubtful significance
2	Minimal	Definite osteophytes but unimpaired joint space
3	Moderate	Moderate diminution of joint space
4	Severe	Joint space greatly impaired with sclerosis of subchondral bone

Table 6-1: Kellgren and Lawrence grading scheme

This is a global scoring scheme which depends heavily upon the osteophyte in classification of the disease, and assumes a linear progression of disease. It also takes no account of differences in OA occurring in different joints. However, OA is a heterogeneous group of diseases displaying various signs and progressing at various rates. No correlation has been found between one diagnostic radiographic sign and another, and no one feature has particularly discriminatory value (Cobby *et al.* 1991) which infers doubt about the suitability of global scoring schemes. This method of grading of OA from radiographs also implies a spectrum of disease progressing in a continuous manner, with the relationship between systems remaining constant. However,

it has been shown that OA is initially a rapidly destructive disease with subsequent slow deterioration, not all joints being active or in phase (Hutton 1989). More recently newer scoring schemes for human radiographic studies have been devised which rely more on individual feature scores and allow for differences between joints. Kallman *et al* (Kallman *et al.* 1989) produced an atlas and scoring system to grade OA of the hand, and compared radiographic grading of individual features, i.e. osteophytes, joint space narrowing, subchondral cysts, subchondral bone sclerosis, lateral deformity and cortical collapse, with the Kellgren and Lawrence (K&L) scheme. Cross-sectional and longitudinal reliability of the grading system was assessed and found to be good, with all features performing well for intra-observer reliability but osteophytes, joint space narrowing and the K&L scheme showing better inter-observer agreement than cysts, deformity and collapse. The individual feature grading scheme compared well with the K&L global scheme. In conclusion this study confirmed that the osteophyte was the best method of defining hand OA. Altman *et al* evaluated methods of grading OA progression of the hand hip and knee (Altman *et al.* 1987) and assessed the contribution of individual joints or joint compartments towards the evidence of radiographic progression as well as the reliability and sensitivity of their method in detecting change. From the results they were able to advise on the most sensitive positioning for radiography of each joint. Their results also indicated that the most useful radiographic variables differed according to joint site and supported the view that there should be different approaches to the evaluation of OA progression at specific joint regions. They recommended that in hip OA joint space narrowing and sclerosis were the most sensitive features to change while in the knee osteophytes were more valuable than joint space narrowing in diagnosing OA. Joint space narrowing however was preferable in detecting OA progression. Recently Altman has produced an atlas of OA change in the hand, hip and knee (Altman *et al.* 1995). Cooper *et al* (Cooper *et al.* 1992) reported on the intra- and inter-observer reproducibility of commonly used radiographic features in assessment of OA of the knee and found that joint space narrowing, osteophytes and bony contour in the tibiofemoral compartment were more reproducible than subchondral sclerosis and cysts.

From these studies it is clear that no single global grading is suitable for the assessment of OA in all joints, and different measures should be given different weights in different circumstances.

<u>Reproducibility studies</u>

It can be seen from the above review that in any scheme it is important to establish the intra- and inter-observer reproducibility of each feature to be scored. The reproducibility of each feature can be significantly affected by the positioning of the joint during radiography, and the extent of weight bearing. It is vital to aim to reduce the variability

caused by such factors. Development of a system for radiographic grading of OA requires knowledge of the reproducibility of individual component features (Cooper *et al.* 1992). Cross-sectional grading of radiographs has been widely studied but longitudinal grading less so (Ravaud *et al.* 1996). Grading of a feature must be reproducible intra- and inter-rater and have good sensitivity to change. It has been shown that training sessions are extremely useful in increasing reproducibility (Altman *et al.* 1987).

No studies of reproducibility in equine OA radiographic grading have been found in the literature. In a canine study investigating stifle OA, global assessments together with osteophytosis and intra-articular mineralisation were found to be more reproducible than subchondral bone sclerosis (Innes 1997).

Current recommendations

In a recent recommendation for assessing progression of OA in the knee and hip joints it was agreed that at present radiography should remain the major outcome measure (Dieppe 1995), while including certain provisions to standardise the technique. Advice on patient positioning for radiography is given and the following recordings suggested:

Hip : Joint space narrowing (JSN) - this should be measured as well as scored on a scale from 0 - 3
: Osteophytes should be graded from 0 - 3
: Subchondral bone changes scored +/-
: Global score should also be included

Knee : As for hip but medial, lateral and patellofemoral joint spaces should be scored separately.

Reproducibility: There should be from 1 - 3 scorers, the radiographs should be blinded, and read centrally. Training and the use of atlases is recommended.

2. Scintigraphy

Scintigraphy is a relatively new imaging method which provides information regarding the physiology, rather than the anatomy of the joint. It is said to be capable of predicting the initiation of structural abnormalities resulting from ongoing pathological activity (Attenburrow *et al.* 1989). Bone scintigraphy depends upon the intravenous circulation of a pharmaceutical agent which has been bound to a gamma emitting radioactive isotope. In equine scintigraphy the most commonly used agent is methylene diphosphonate which is labeled with Technetium^{99m}. Methylene diphosphonate is a salt which has a high affinity

for hydroxyapatite crystals in bone and therefore it preferentially binds to those areas of bone undergoing active metabolic change. The radioactivity level or "counts" emitted from an area of bone are detected by use of a gamma camera and so the concentration and distribution of the isotope can be assessed. It has been recognised for many years that bone tracers localise to OA joints (Bauer *et al.* 1961). Studies in man (Hoffer *et al.* 1976; Hutton *et al.* 1986) and animals (Christensen 1985; Metcalf 1985; Ueltschi 1977) have shown scintigraphy to be a very sensitive technique in joint disease, although not as specific as radiography. It is possible to detect only 10^{-13} g radiopharmaceutical, but bony lesions can only be detected by radiography when destruction/formation of bone occurs in grams (Devous *et al.* 1984). Radioisotope uptake depends on osteoblastic activity, and also blood flow and vascular permeability. The "hot" areas of increased isotope uptake seen on a bone scan are a consequence of the metabolic response to osteoblastic activation and can therefore be seen within hours of a bone response (O'Callaghan 1991). Scintigraphy has been used in horses for the investigation of occult lameness increasingly during the last 20 years (Ueltschi *et al.* 1977; Weaver 1995). The advantage of this imaging method is that subchondral bone changes may be diagnosed before radiological signs of OA can be detected. In man it has been found that scintigraphy can be predictive of OA progression (Dieppe *et al.* 1993) in the knee and in the hand (McCarthy 1994).

In equine practice scintigraphy has become a popular diagnostic aid in cases of lameness that can not be localised by other methods. It is the method of choice for imaging the spine and pelvis i.e. regions that are difficult to examine radiographically, and protocols for the assessment of navicular disease by scintigraphy are being developed. At present scintigraphy is not commonly used as a primary method to diagnose OA.

Radioisotope uptake has been found to have a good correlation with radiographic data in the dog (Innes *et al.* 1996) but can also provide additional information. The pattern of isotope uptake in OA has been found to be important (McCrae *et al.* 1992). In man it has been reported (McCrae *et al.* 1992) that a tramline pattern may indicate osteophytes at the joint margins, a linear pattern of uptake was indicative of sclerosis in later stage OA, while the more generalized pattern of uptake related to early OA. In the dog the marginal pattern of uptake in stifle OA was associated with instability of the joint, osteophytes, and intra-articular mineralisation (Innes *et al.* 1996).

It is important to appreciate that the relative uptake of isotope by bone may be affected by various factors other than that of increased metabolic activity. An alteration in blood supply may affect the distribution, as can variations in the age or work of the animal (Devous and Twardock 1984).

No reports have been found in the equine literature concerning the grading of scans. In a canine study (Innes *et al.* 1996) however, scans of cases with stifle OA were assessed using a three grade score of isotope uptake and a two grade score of pattern, and the reproducibility of this technique was found to be good.

3. Scintimetry

Scintimetry is the name given to the semiquantitative analysis of changes detected on scans. This allows comparison of scans and correlation of scintigraphy results with other measures. The quantification of bone scans remains problematic with positioning, dose of radioisotope, and the counts measured having the potential to cause considerable variability between scans. For this reason scintimetry has not been commonly used as a marker of change in OA trials. The most commonly used method is to measure the ratio of counts at the OA joint relative to a local reference bone site (Metcalf 1985; Olsen *et al.* 1988). There are many references to the use of scintigraphy in the investigation of equine lameness but few which have used scintimetry in their analysis of longitudinal data. In 1993 Todhunter (Todhunter *et al.* 1993) used scintimetry measurements over a period of 17 weeks to evaluate the effects of exercise and intra-articular medication on joints with experimentally induced carpal cartilage defects.

Relationship between imaging and clinical outcome

Despite radiography being accepted as the gold standard measure of outcome in OA in man, it is rare to find reports of significant correlation between radiological and clinical signs in the literature. In the Bristol "OA500" study, a 3 year study involving 415 patients with knee OA, there was no correlation between radiographic and clinical changes. It was concluded that clinical and radiographic changes may not be in phase with each other in the progression of OA and that radiographic change may not be a good surrogate for clinical outcome in established OA (Dieppe *et al.* 1997). In a canine stifle OA study, scintigraphy was found to be associated with clinical findings, but neither scintigraphy nor radiography were predictive of change in disability score (Innes 1997). In a study of synovitis in the human proximal interphalangeal joint, changes in scintimetry indices were found to correlate with clinical changes (Olsen *et al.* 1988).

Aims

1. Comparison of diagnosis of equine OA by radiography and scintigraphy.

2. Evaluation of the reliability of the radiographic scoring scheme.

3. Evaluation of the reliability of the scintigraphy scoring scheme.

4. Assessment of the use of scintimetric quantitative analysis of scans.

5. Relationship of scintigraphy patterns with radiographic changes.

6. Correlation of radiography and scintigraphy with clinical changes.

7. Use of radiography and scintigraphy as measures of outcome in the CaPPS trial.

Methods

Radiography :

On each examination each horse underwent radiography of both the clinically active osteoarthritic and the contralateral joint. Horses were radiographed in the standing position under light sedation. On the first examination this was done using four standard views of the joint i.e. dorsopalmar, lateromedial, dorsolatero-palmaromedial oblique and palmerolateral-dorsomedial oblique. On the three subsequent examinations the views that had been found most useful in highlighting the pathology were repeated each time. On the first examination, in order to make a diagnosis of osteoarthritis, the radiographs were examined for signs of narrowing of the joint space, osteophytosis, subchondral bone changes, and soft tissue swelling indicative of synovial effusion or joint capsule thickening.

Quantification :

The following scoring system was devised (Table 6-2):

Feature	Score			
Joint space narrowing	0	1		
Osteophytosis	0	1	2	3
Subchondral bone sclerosis	0	1		
Subchondral bone erosions	0	1		
Global score	0	1	2	3

Table 6-2: Radiographic scoring system

The radiographs were blinded to case identification and to date order. Three experienced veterinary radiologists were then asked to examine and score each affected OA joint, and its contralateral, at each of the four time points of the trial. Inter-observer variation was then calculated from these scores using the kappa statistic. One radiologist was asked to score ten sets of radiographs again after an interval of 14 days. The resulting second set of scores allowed intra-observer variation to be calculated.

Scintigraphy:

Materials:

Metomidine - "Domosedan" Pfizer Ltd., Sandwich, Kent.

Gamma camera - "Maxicamera" IGE Medical systems., Slough, Berkshire.

Tc99mMDP - Radiopharmacy, Bristol General Hospital.

MAPS 2000 computer

MAPS 5000 image processing software - Link Medical Ltd., Marlow, Buckinghamshire.

Nuclear Diagnostics computer

Methods:

Bone phase scans were acquired of the clinically osteoarthritic and the contralateral joint from each horse on the first and third i.e. 0 and 6 month visit. On the first visit the whole of the lame leg was scanned to check for any other abnormalities. Each horse was injected intravenously with 10Mbq/kg Technetium ^{99m} methylene diphosphonate ($TC^{99m}MDP$). After three hours, horses were sedated with detomidine hydrochloride (Domosedan Pfizer Ltd) 10 µg/kg and led from the isolation stable to the gamma camera room where they were positioned to stand in stocks. Using a low resolution collimator and acquiring between 50000 counts for metacarpophalangeal (MCP), proximal interphalangeal (PIP),

and distal interphalangeal (DIP) joints, and 75000 for midcarpal (MC) and tarsometatarsal (TMT) joints, lateral images were acquired from each joint. In cases of DIP joint OA, solar views were also acquired by placing the affected foot on top of the gamma camera. Images were stored both on the computer and on xray film. The counts measured and the time taken to collect the image were recorded on each occasion, taking care to standardise the number of counts collected for each horse on subsequent occasions.

Scoring scheme:

A scoring scheme (Table 6-3) was devised to quantify the degree and pattern of uptake of isotope for each joint on each occasion. Four veterinary surgeons experienced in scintigraphy were asked to score all 19 sets of scans both of the OA and contralateral joint. The scores were then analysed for inter-observer variation by the kappa statistic. One observer (CF) scored all the scans on two occasions at an interval of 14 days in order to allow intra-observer variation to be measured.

Feature	Score			
Degree of isotope uptake	0	1	2	3
Pattern	Normal	Linear	Generalised	
Global score	0	1	2	3

Table 6-3: Scintigraphic scoring scheme

Scintimetry

The second method of quantification of the scans was by measuring the counts in a given region of interest drawn onto the computer generated images (Figure 6-1). On each image, a line was drawn on the computer image to encompass the "hot" area of greatest isotope uptake around the joint. This region was labeled "A". Another region, "B" was drawn to enclose as much as possible of the diaphyseal bone proximal to the joint. The number of counts per area A and B were then calculated by the computer. Comparison of these two values i.e. A/B indicated an activity index (AI) for the joint i.e. the degree of increased uptake of isotope around the joint as compared to that in the neighbouring reference bone (Figure 6-1). The activity index for both the clinically affected OA joint and the contralateral joint were calculated on each occasion. To measure the reproducibility of this method, 10 images were re-measured after an interval of 14 days and the resulting intraclass correlation coefficient (ICC) calculated.

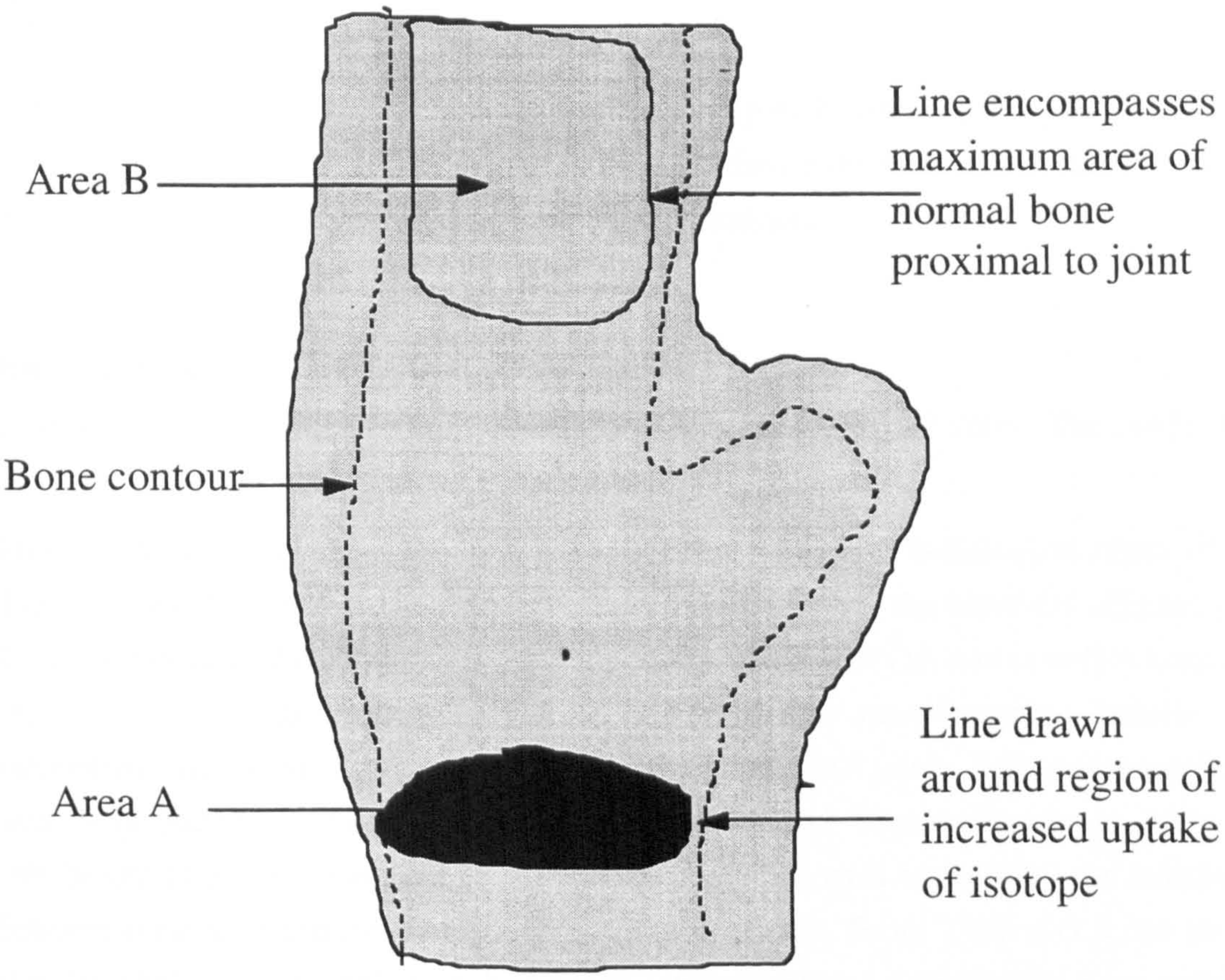

Figure 6-1: Schematic diagram to demonstrate calculation of activity index in a case of osteoarthritis of the intertarsal joints (bone spavin)

Statistics

To investigate reproducibility the agreement between measurements was calculated using the kappa statistic (κ) (Fleiss 1981). A κ value of below 0.4 was considered unacceptable and a κ value of above 0.75 considered excellent agreement. Reproducibility of scintimetric AI measurement was measured using the intraclass correlation coefficient (ICC) (Deyo *et al.* 1991). For further discussion of these measurements see Chapter 5.

For comparison of group outcome the Wilcoxon signed rank test was used for nonparametric results, the paired students t test for parametric results, and Fishers exact test for dichotomous variables.

Spearmans correlation coefficient (r_s) was used for correlation of nonparametric and Pearsons correlation coefficient (r_p) for correlation of parametric variables.

An estimate of the sensitivity of outcome measures was made where appropriate with the standardized response mean.

Results

For demographic variables relating to the study group of horses see Chapter 4. Horse 11 was withdrawn from the clinical trial following the first examination and therefore results from this case were not included in the imaging analysis.

Radiography

Paired radiographs from 19 horses were available for scoring at entry. The results from these are shown in Table 6-4.

From these radiographs, 17/19 horses and 28/38 joints had radiological signs of OA. Three horses (5, 8, and 19) had no radiologic OA changes in the clinically affected joint. 11/19 horses had bilaterally symmetrical radiological signs of OA and in only 6 horses (4, 5, 7, 15, 17, 20) were the changes unilateral. The most common features were osteophytes which were detected in 27/28 radiologically OA joints. Joint space narrowing was only judged to be present in 8/28, subchondral bone sclerosis in 2/28, and subchondral bone erosions in 5/28 OA joints. Only 2 horses showed all the radiological features to be scored in the clinically affected joint (6,13). From Table 6-5 it can be seen that the TMT joints scored more highly for osteophytes and subchondral bone erosions, and received the higher global scores for radiological OA than the other joints. Bilateral changes were most commonly seen in the PIP joints.

Radiography as a measurement of outcome in the trial

Two horses in the low dose (1 and 20) and 1 horse (4) in the high dose group were judged to show radiological improvement in the clinically affected joint during the 9 months of the trial. No horses showed radiological deterioration and 16 remained unchanged.

There was no significant difference (Students-t test) between the treatment groups for radiological global scores at the beginning or end of the trial, and no significant difference in change in global radiological score (Fishers exact test).

Horse	Lame leg	Joint	Radiographic changes detected at initial examination									
			Limb		JSN		Osteophytes		SBS		SBE		Global score	
1	LH	TMT	LH	RH	0	0	2	1	0	0	1	0	2	1
2	RF	PIP	LF	RF	0	0	1	1	0	0	0	0	1	1
3	LF	PIP	LF	RF	1	1	1	2	0	0	0	0	1	1
4	RF	DIP	-	RF	0	0	0	1	0	0	0	0	0	1
5	RH	TMT	LH	-	0	0	1	0	0	0	0	0	1	0
6	RH	TMT	LH	RH	1	1	1	2	0	1	0	2	1	3
7	RF	MC	-	RF	0	0	0	1	0	0	0	0	0	1
8	RF	DIP	-	-	0	0	0	0	0	0	0	0	0	0
9	RF	DIP	LF	RF	0	0	1	1	0	0	0	0	1	1
10	RH	PIP	LH	RH	0	0	1	1	0	0	0	0	1	1
12	LH	PIP	LH	RH	0	0	1	1	0	0	0	0	1	1
13	RH	TMT	LH	RH	0	1	1	2	0	0	0	1	1	2
14	RF	PIP	LF	RF	1	1	1	1	0	0	1	0	1	1
15	RF	MCP	-	RF	0	1	0	0	0	0	0	1	0	1
16	RF	DIP	LF	RF	0	0	1	1	0	0	0	0	1	1
17	RH	TMT	-	RH	0	0	0	1	0	0	0	0	0	1
18	LF	PIP	LF	RF	0	0	1	1	0	0	0	0	1	1
19	RF	DIP	-	-	0	0	0	0	0	0	0	0	0	0
20	RF	DIP	-	RF	0	1	0	1	0	1	0	0	0	1

Table 6-4: Scores of radiographic features of all joints showing OA changes from all horses at initial examination

Key : JSN = Joint space narrowing, SBS = subchondral bone sclerosis, SBE = subchondral bone erosions,

(-) indicates no signs of OA within joint

For scoring system see Methods - scoring of radiographs

Joint space narrowing		**0**	**1**			**Mean**
Joint affected (Jts/horses)	TMT (8/5)	**2** *(3)*	**2** *(1)*			**0.5** *(0.25)*
	MC (1/1)	**1**				**0**
	MCP (1/1)		**1**			**1**
	PIP (12/6)	**4** *(4)*	**2** *(2)*			**0.33** *(0.33)*
	DIP (6/6)	**3** *(2)*	**1**			**0.25** *(0)*

Osteophytes		**0**	**1**	**2**	**3**	**Mean**
Joint affected (Jts/horses)	TMT (8/5)		**2** *(3)*	**3**		**1.6** *(1)*
	MC (1/1)		**1**			**1**
	MCP (1/1)	**1**				**0**
	PIP (12/6)		**6** (5)	*(1)*		**1** *(1.2)*
	DIP (6/6)		**4** *(2)*			**1** *(1)*

Subchondral bone sclerosis		**0**	**1**		**Mean**
Joint affected (Jts/horses)	TMT (8/5)	**3** *(4)*	**1**		**0.25** *(0)*
	MC (1/1)	**1**			**0**
	MCP (1/1)	**1**			**0**
	PIP (12/6)	**6** *(6)*			**0** *(0)*
	DIP (6/6)	**3** *(2)*	**1**		**0.25** *(0)*

Subchondral bone erosions		**0**	**1**	**2**		**Mean**
Joint affected (Jts/horses)	TMT (8/5)	**1** *(4)*	**2**	**1**		**1** *(0)*
	MC (1/1)	**1**				**0**
	MCP (1/1)		**1**			**1**
	PIP (12/6)	**6** *(5)*	**1**			**0.14** *(0)*
	DIP (6/6)	**4** *(2)*				**0**

Global score			**0**	**1**	**2**	**3**	**Mean**
Joint affected (Jts/horses)	TMT (8/5)			**1** *(4)*	**2**	**1**	**2** *(1)*
	MC (1/1)			**1**			**1**
	MCP (1/1)			**1**			**1**
	PIP (12/6)			**6** *(6)*			**1** *(1)*
	DIP (6/6)			**4** *(2)*			**1** *(1)*

Table 6-5: Summary of numbers of scores achieved for each feature, plus mean scores, in different OA joints.

All 28 joints found to have radiographic signs of OA included, clinically active in bold and contralateral in bracketed italics.

Feature		Raters			
		MC v AB	MC v BB	AB v BB	Overall
Joint space narrowing	κ	0.32	0.22	**0.48**	**κ = 0.48**
	95%CI	+/- 0.33	+/- 0.33	**+/- 0.01**	**+/- 0.01**
Osteophytes	κ	**0.61**	**0.43**	**0.53**	**κ = 0.52**
	95%CI	**+/- 0.09**	**+/- 0.08**	**+/- 0.07**	**+/- 0.06**
Subchondral bone erosions	κ	0.17	0.38	0.12	κ = 0.19
	95%CI	+/- 0.22	+/- 0.18	+/- 0.13	+/- 0.18
Subchondral bone sclerosis	κ	0.28	0.45	0.38	κ = 0.42
	95%CI	+/- 0.52	+/- 0.22	+/- 0.51	+/- 0.19
Global score	κ	**0.62**	0.37	**0.51**	**κ = 0.47**
	95%CI	**+/- 0.09**	+/-0.07	**+/- 0.08**	**+/- 0.05**

Table 6-6: Inter-rater reliability (cross-sectional) of radiological scoring (unweighted kappa values)

(Kappa values and confidence intervals indicating good agreement highlighted in bold)

Reproducibility of scoring method

Inter-observer reproducibility

Cross-sectional agreement

The kappa values for cross-sectional reproducibility of the different radiographic features scored are shown in Table 6-6. Scoring of JSN, osteophytes and global score was acceptably reproducible. Although the kappa value for subchondral bone sclerosis was above the minimum acceptable value of 0.4, the confidence interval was unacceptably large.

There were no disagreements of more than 1 grade therefore unweighted kappas were used.

Longitudinal agreement

The inter-observer agreement for longitudinal changes in radiographic scores was unacceptable for all features e.g.

JSN $\kappa = 0.26$

Osteophytes $\kappa = 0.38$

Global $\kappa = -0.08$

It was not possible to check the responsiveness of the scoring system since this can only be done by using the system to score the response to an intervention of known outcome. However to provide an indication of the sensitivity of the system, the standard response mean was calculated i.e. Mean value of score change between radiographs at beginning and end of the trial / Standard deviation of that change. For all features and for the global score the SRM was unreasonably low. However rather than indicating a failing of the scoring system, this may have been because of the short duration of the trial and the slow progression of radiological change.

<u>Relationship between radiology and clinical outcome</u>

There was no correlation between the clinical global score of improvement in lameness of the horses with the change in radiological global score throughout the trial (Table 6-7).

Group A - Low dose							Group B - High dose						
Horse	OA joint	Global clinical improvement	Global radiological scores (clinically OA joint highlighted)				Horse	OA joint	Global clinical improvement	Global radiological scores (clinically OA joint highlighted)			
			L1	L4	R1	R4				L1	L4	R1	R4
1	TMT	2	**2**	**1**	*1*	*0*	5	TMT	1	*0*	*1*	**0**	**0**
13	TMT	-1	*1*	*1*	**2**	**2**	17	TMT	1	*0*	*0*	**1**	**1**
							6	TMT		*1*	*0*	**3**	**3**
3	PIP	2	**1**	**1**	*1*	*1*	2	PIP	1	*1*	*1*	**1**	**1**
10	PIP	1	*1*	*1*	**1**	**1**	12	PIP	0	**1**	**1**	*1*	*1*
18	PIP	1	**1**	**1**	*1*	*1*	14	PIP	1	*1*	*1*	**1**	**1**
8	DIP	2	*0*	*0*	**0**	**0**	9	DIP	1	*1*	*1*	**1**	**1**
16	DIP	1	*1*	*1*	**1**	**1**	19	DIP	-1	*1*	*0*	**0**	**0**
20	DIP	1	*0*	*1*	**1**	**0**	4	DIP	0	*0*	*1*	**1**	**0**
7	MC	-1	*0*	*1*	**1**	**1**	15	MCP	0	*0*	*0*	**1**	**1**

Table 6-7: Comparison of longitudinal changes in radiographic scores and global score of lameness improvement during trial.

Horse numbers arranged in matched pairs.
Key : L1 = Score assigned to left limb radiograph at entry, R1 = Score assigned to right limb radiograph at entry
L4 = Score assigned to left limb radiograph at end of trial, R4 = score assigned to right limb radiograph at end of trial.

Scintigraphy

Paired scintigraphical bone scan films were available for scoring from 19 horses at entry to the trial and at the third examination 6 months later. Of these cases 17/19 horses and 32/38 joints were found to have positive scintigraphy changes based on increased uptake of isotope in one or more joints at entry. In 14/17 horses the scintigraphical changes seen were bilateral (Table 6-8).

It can be seen that the global score (Table 6-9) closely reflected the score of uptake of isotope and was not affected by the pattern of isotope distribution. Scintigraphical changes in the TMT and the PIP joints were bilateral and the uptake (and global) scores for these joints tended to be higher than for the other joint types. It was also more common to find a linear pattern of isotope uptake in the TMT and PIP joints.

Group A - Low dose							Group B - High dose						
Horse	OA joint	Global clinical improvement	Global scintigraphy scores (clinically OA joint highlighted)				Horse	OA joint	Global clinical improvement	Global scintigraphy scores (clinically OA joint highlighted)			
			L1	L4	R1	R4				L1	L4	R1	R4
1	TMT	2	**2**	**2**	*1*	*0*	**5**	TMT	1	*2*	*1*	**2**	**0**
13	TMT	-1	*2*	*1*	**2**	**1**	**17**	TMT	1	*1*	*1*	**2**	**2**
							6	TMT		*1*	*1*	**3**	**3**
3	PIP	2	**2**	**2**	*2*	*2*	**2**	PIP	1	*1*	*2*	**1**	**2**
10	PIP	1	*1*	*2*	**2**	**2**	**12**	PIP	0	**2**	**1**	*2*	*0*
18	PIP	1	**1**	**1**	*1*	*1*	**14**	PIP	1	*1*	*2*	**1**	**1**
8	DIP	2	*1*	*1*	**1**	**0**	**9**	DIP	1	*0*	*0*	**0**	**1**
16	DIP	1	*1*	*0*	**1**	**0**	**19**	DIP	-1	*1*	*1*	**1**	**1**
20	DIP	1	*0*	*0*	**0**	**1**	**4**	DIP	0	*1*	*1*	**1**	**1**
7	MC	-1	*0*	*1*	**1**	**2**	**15**	MCP	0	*0*	*1*	**1**	**1**

Table 6-8: Comparison of longitudinal changes in scintigraphic scores and global score of lameness improvement during trial.

Horse numbers arranged in matched pairs.
Key : L1 = Score assigned to left limb scan at entry, R1 = Score assigned to right limb scan at entry
L4 = Score assigned to left limb scan at end of trial, R4 = score assigned to right limb scan at end

Scintigraphy as measurement of change in trial

There was no significant difference between high and low dose groups for uptake in lame or sound limbs at the beginning or end of the trial and therefore no significant difference in the change in uptake between the beginning and end of the trial.

Uptake Score		**0**	**1**	**2**	**3**	**Mean**
Joint affected (**Jts**/horse)	TMT (**10**/5)		*(3)*	**4** *(2)*	**1**	**2.2** *(1.4)*
	MC (**1**/1)	*(1)*	**1**			**1***(0)*
	MCP(**1**/1)	*(1)*	**1**			**1***(0)*
	PIP (**12**/6)		**3** *(3)*	**3** *(3)*		**1.5** *(1.5)*
	DIP (**8**/6)	**2** *(2)*	**4** *(4)*			**0.67** *(0.67)*

Pattern of isotope distribution	**Normal**	**General**	**Linear**		
Joint affected (**Jts**/horse)	TMT (**10**/5)	*(2)*	**1** *(3)*	**4**	
	MC (**1**/1)	*(1)*	**1**		
	MCP(**1**/1)	*(1)*		**1**	
	PIP (**12**/6)		**1** *(0)*	**5** *(6)*	
	DIP (**8**/6)	**2** *(2)*	**2** *(1)*	**1** *(2)*	

Global score		**0**	**1**	**2**	**3**	**Mean**
Joint affected (**Jts**/horse)	TMT (**10**/5)		**3**	**4** *(2)*	**1**	**2.2** *(1.4)*
	MC (**1**/1)	*(1)*	**1**			**1** *(0)*
	MCP(**1**/1)	*(1)*	**1**			**1** *(0)*
	PIP (**12**/6)		**3** *(4)*	**3** *(2)*		**1.5** *(1.33)*
	DIP (**8**/6)	**1** *(3)*	**4** *(4)*			**0.8** *(0.6)*

Table 6-9: Summary of numbers of scores achieved for each feature, plus mean scores, in different OA joints at entry. All 32 joints found to have scintigraphic signs of OA are included, clinically active in bold and contralateral in bracketed italics.

Reproducibility.

Weighted kappas were used for the measurement of isotope uptake where the discrepancies in agreement between raters were more than one grade (Table 6-10).

Intra-observer variation:

Cross-sectional:

The cross-sectional intra-rater reliability for scoring pattern and uptake was considered acceptable :

Pattern (unweighted) $\kappa = 0.54$ (95% CI = 0.39 - 0.69)

Isotope uptake (weighted) $\kappa = 0.63$ (95% CI = 0.49 - 0.76)

Longitudinal:

The longitudinal reliability of the scoring scheme, measured by unweighted kappas on the global changes between the first and second scan was also good:

$\kappa = 0.58$ (95% CI = 0.51 - 0.65)

Inter-observer variation:

Cross-sectional:

Raters	MC v AB	MC v BB	MC v CF	BB v CF	AB v CF	BB v AB
Disagree-ments	1	10	3	6	7	22

Table 6-10: No of inter-rater disagreements in score of more than one grade

The inter-rater reliability was unacceptably low with an overall kappa value of

Pattern (unweighted) $\kappa = 0.23$ (95% CI = 0.18 - 0.27)

Isotope uptake(weighted) $\kappa = 0.37$, (95% CI = 0.32 - 0.42) (Tables 6-11, 6-12)

Global scoring closely reflected uptake scoring for all except one rater (BB).

Rater 1	Rater 2	Unweighted kappa	95%CI
MC	AB	0.37	0.25-0.49
MC	BB	0.17	0.05-0.21
MC	CF	0.46	0.32-0.60
BB	CF	0.08	0.01-0.16
AB	CF	0.26	0.21-0.31
BB	AB	0.16	0.12-0.21

Table 6-11: Inter-rater kappas (unweighted) for pattern scoring

Rater 1	Rater 2	Weighted kappa	95%CI
MC	AB	0.50	0.38-0.62
MC	BB	0.33	0.21-0.46
MC	CF	0.53	0.39-0.66
BB	CF	0.34	0.20-0.48
AB	CF	0.35	0.24-0.46
BB	AB	0.24	0.15-0.34

Table 6-12: Inter-rater kappas (weighted) for uptake scoring

There was also overall poor cross-sectional agreement inter-rater on a simplified scale of normal or abnormal uptake.

Longitudinal agreement:

The longitudinal inter-observer reliability of the scheme was measured using unweighted kappas on a simplified score of improvement v no improvement between first and second scans. For this measurement the agreement between two raters (CF and MC) was considered excellent ($\kappa = 0.75$) , but the agreement between every other combination of the four raters was unacceptably low.

Analysis was therefore made on the results of one rater (CF) for which the intra-rater reliability was reasonable.

Cross-sectional difference in scan uptake between lame and contralateral joint

The difference in uptake between sound and lame limbs at the beginning of the trial was not quite significant (p=0.06 Paired student t test). There was a significant correlation between the scores of scan uptake in the clinically active and contralateral joints, i.e.

$r_p = 0.64$, 95% CI = 0.26 - 0.85 p = 0.002

Correlations between scintigraphy and radiology

There were significant positive correlations between the radiographic global score of OA severity and the scan radioisotope uptake score , i.e.

$r_p = 0.46$ 95% CI = 0.16 - 0.68 p = 0.004

However there was no longitudinal correlation between the change in global radiography score and the change in scan uptake score between the first and second scan (6 months interval).

Association between scintigraphy score and radiological pattern.

The correlation between increasing osteophyte score and linear pattern was not quite significant

$r_s = 0.44$, 95% CI = 0.03 - 0.75 p=0.06.

There was no correlation between duration of lameness and generalised or linear pattern.

Relationship between scintigraphy and clinical outcome

There was also no correlation between the change in uptake of the scans and the global score of clinical lameness improvement.

Scintimetry

Computer images for 19 horses at entry to the trial were analysed quantitatively. Images for only 18 of these were available at the third examination, i.e. 6 months later.

Joint	n	Lame 1	Contra 1	Lame 6	Contra 6
	Group A - Low dose				
TMT	2	1.85	2.34	2.04	1.51
MC	1	5.8	2.67	2.24	1.88
PIP	3	2.23	1.96	1.66	1.54
DIP	3	1.96	2.30	1.99	1.86
	Group B - High dose				
TMT	3	2.38	2.39	1.84	2.11
MCP	1	1.16	1.23	2.11	1.99
PIP	3	2.03	1.83	1.87	2.02
DIP	3	2.09	1.64	1.46	1.41

Table 6-13: Mean activity indices for different joints in both treatment groups at first and second scans

Lame 1 = AI from clinically active OA joint at first scan
Contra 1 = AI from contralateral joint at first scan
Lame 6 = AI from clinically active OA joint at second scan
Contra 6 = AI from contralateral joint at second scan

Joint	n	Lame 1	Contra 1
TMT	5	2.17	2.04
PIP	6	2.13	1.89
DIP	6	2.03	1.97
MC	1	5.8	2.67
MCP	1	1.16	1.23

Table 6-14: Overall mean AI values for different joints at entry to trial.

Scintimetry as measurement tool of trial

There was no significant difference in activity indices (AI) of the clinically OA joint between treatment groups A and B at the beginning or end of the trial.

There was no significant difference in AI values between different joints at entry to the trial.

Reproducibility

The reliability of this method of quantitation of the images, calculated by intraclass correlation coefficient (ICC) = 0.71. This was considered good .

Difference in AI between lame and contralateral joint.

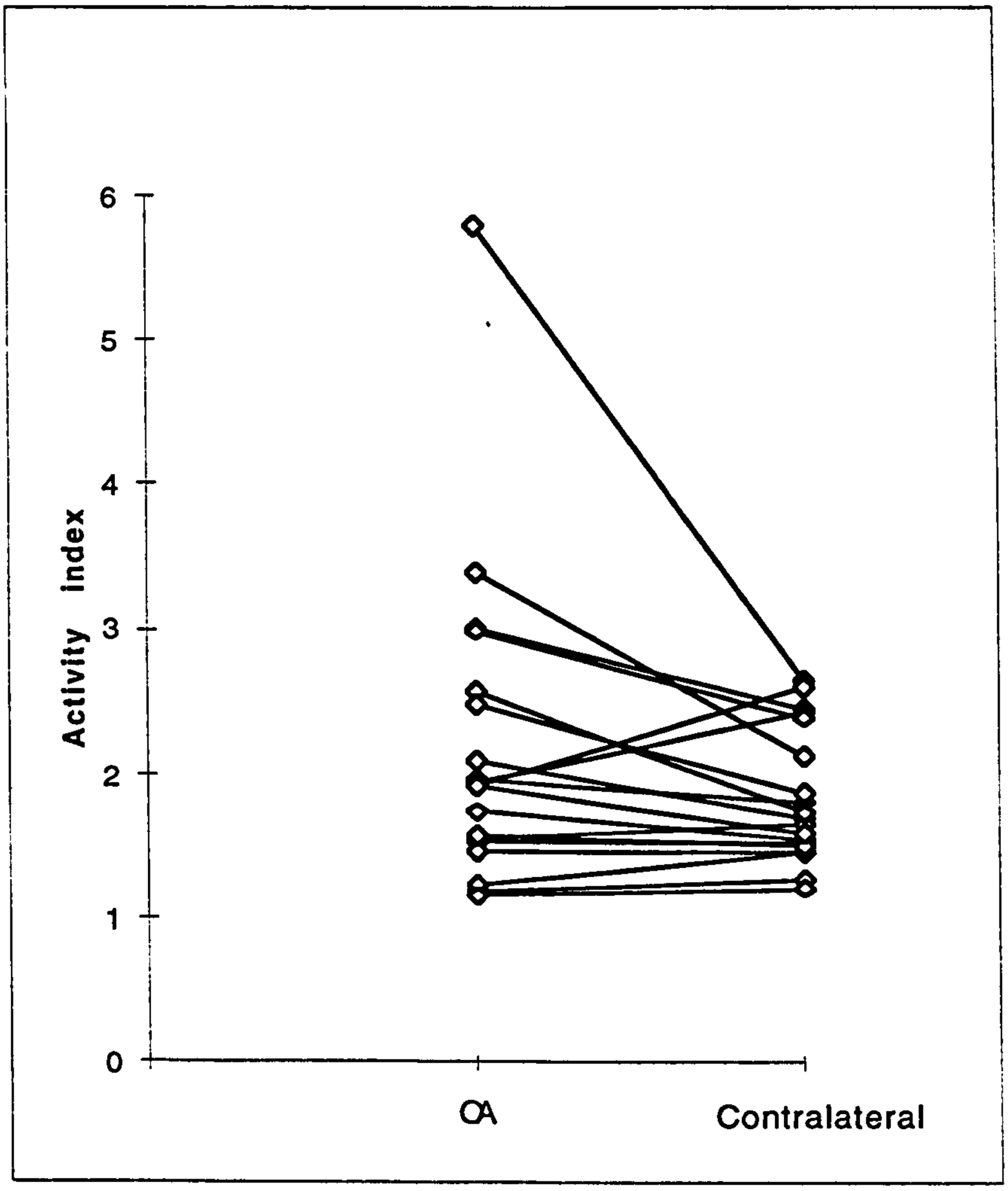

Figure 6-2: Comparison between activity index of clinically active OA joint and contralateral.

There was no significant difference in AI between the clinically active OA joint and the contralateral, but this may have been because in 82 % horses the disease was symmetrically bilateral. The correlation between AI values from the lame and contralateral limbs was significant however :

r = 0.59 (95%CI = 0.19 - 0.83) p = 0.007 n = 19

This result indicates that there may have been a systemic effect affecting the AI values. There was no significant difference between the normal reference bone in the active (mean 15.19 counts / area) and the contralateral (mean 15.85 counts/area) limbs.

Correlation between scintigraphy and scintimetry

There was a significant positive correlation (Spearmans coefficient -r_s) between the scan score of uptake and the activity index of the joint .

$r_s = 0.32$ 95% CI = 0.08 - 0.52 p = 0.006 n=74

There was no significant longitudinal correlation between the change in uptake score from the scans and change in activity index.

The score for linear pattern uptake was only weakly correlated with activity index of the joint:

$r_s = 0.24$ 95% CI = 0.03 - 0.45 p = 0.04 n=74

Correlation between scintimetry and radiology

There was no significant correlation (Spearmans) between the activity indices and the radiological scores nor was there any correlation between the change in AI and the change in radiology scores.

Correlation with age or duration of lameness.

There was no correlation between AI and age of the horse, nor duration of lameness.

Correlation between scintimetry and clinical outcome

Using the non parametric Spearmans coefficient the correlation between the global score of change in lameness throughout the trial and the change in AI of the clinically active OA joint was just significant (Figure 6- 3)

r_s =0.47 95%CI = - 0.01 - 0.77 p=0.049

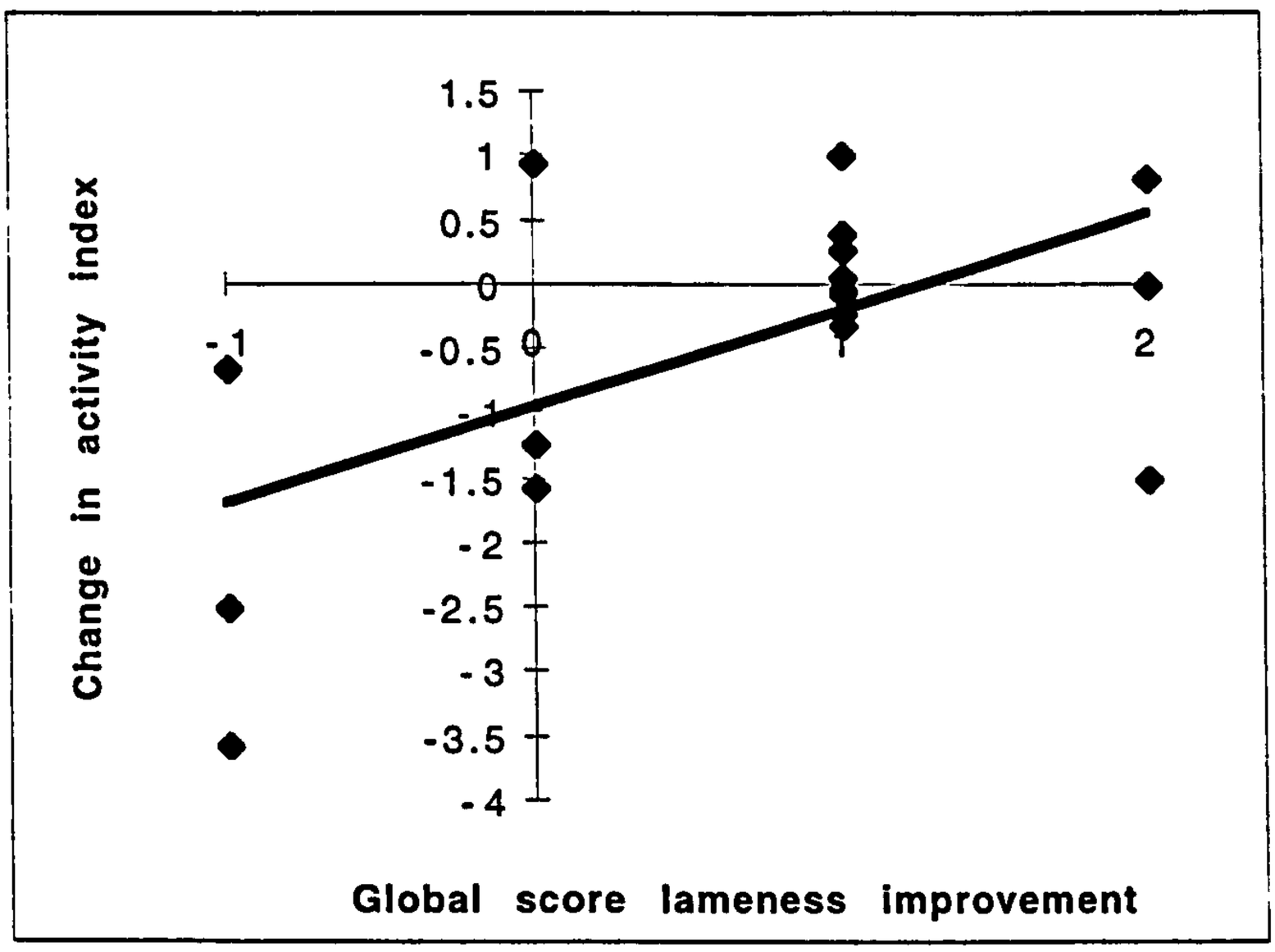

Figure 6-3: Correlation between global score of clinical improvement and the change in activity index of the clinically OA joint throughout the trial.

Discussion

Radiography

In this study the scoring scheme used was found to be reliable cross-sectionally both intra- and inter-rater for scoring joint space narrowing, osteophytes and for global score. Radiography has been validated in human trials, but no such reports could be found in the equine literature. Although radiography is generally accepted as the gold standard method of assessment of OA in equine medicine, its validation is fundamental to its use in the accurate assessment of OA in equine clinical trials.

No difference in outcome between the two treatment groups in the CaPPS trial was demonstrated by radiography. However, as discussed in chapter 4, the sample size of the trial was too low to provide adequate power and therefore the chances of Type II error were increased. Outcome is also affected by the validity of the measurement tool. The method of scoring used in this study was reliable cross sectionally both intra- and inter-rater for joint space narrowing, osteophytes and for global score. The fact that subchondral bone changes were not found to be reliable could have been affected by the low numbers of OA joints in which these features were noted (< 18%). Longitudinally however, the scoring scheme was not found to be reliable and only 3 horses showed any longitudinal change in radiographic score during the 9 months of the trial. Apart from the obvious possibility that the scoring system may not have been sensitive enough to detect change, this could have been because of the short duration of the trial. It has been stated in the recommendations for clinical trials (Altman 1990) that any trial in which imaging methods are used should be at least 1 year in duration, and in fact Ravaud recommends 2-3 years (Lequesne *et al.* 1994) for radiographic studies. It may also be that in the population of clinical cases of OA that were used in this trial 16/19 horses had lameness of more than 3 months duration and could therefore be considered to have chronic OA changes. It has been noted previously (Ravaud *et al.* 1996) that in the later stages of OA, scoring systems such as the K&L scheme, i.e. dependent on the osteophyte, may reach a plateau, since if scored highly at onset of the trial these schemes do not allow for further changes. However despite the fact that the global scoring scheme ranged from 0 - 3, only one horse was scored as 3 (Horse 6), and two horses scored 2 (Horses 1 and 13) at entry to the trial. Of all the other cases, scoring 0 or 1, no cases increased their score during the trial i.e. showed worsening radiological signs of OA. This finding therefore seems to contradict the theory that the chronicity of the disease had caused a ceiling effect in the scoring system, and indicates that it is more likely that too short a duration of the trial was the reason for the lack of detection of radiological changes.

Another factor which may have affected the reliability of longitudinal scoring was the method of reading the radiographs. In this study all the radiographs were read blinded to date order, but it has been recommended (Ravaud *et al.* 1996) that when assessing longitudinal change visualising the two sequential radiographs together would improve assessment of longitudinal change where changes are slight.

Some differences in radiological features commonly detected were found between different joints, osteophytes being scored more highly in the TMT joints for example. Subchondral bone erosions have been said to be most common in the tarsal joints - in this study 3 out of the 5 joints scored as having erosions present were TMT joints. Although subchondral bone sclerosis is regarded as one of the primary OA signs it was only noted in 2/28 OA joints in this study. It seems likely that for adequate scoring of radiological changes different features should be scored according to joint but a larger sample size would be necessary to investigate this further with stricter control over the duration of OA in each joint.

The scoring system used in this study is similar to the Kellgren and Lawrence scheme in that the osteophyte seems to have the most influence over the global score. Osteophytes were found to be very common, being scored as present in 27/28 OA joints.

Scintigraphy

Scintigraphy was useful in the diagnosis of OA in cases where there were no radiological signs of OA. However, although the scoring system used here to quantify the degree of OA was found to be reliable intra-observer, it was unreliable inter-observer both cross-sectionally and longitudinally.

No difference in outcome between the two treatment groups in the CaPPS trial was demonstrated by scintigraphy. The reason for this may be, as previously mentioned, because of the inadequate sample size of the study. However, it could also be in this case because the scoring system was found to be unreliable inter-observer. It was surprising that there was not even agreement as to the normality or abnormality of the scans. A training session as such was not used in this study, which in retrospect could have been a cause of poor reproducibility, but since each rater was commonly involved in assessing scintigraphs this had not been considered necessary at the time. It was interesting that two raters showed good agreement throughout (MC and CF) while one rater showed consistently poor agreement - this may indicate a difference of opinion in the interpretation of the scans rather than a failing of the scoring system itself. Scintigraphical bone scanning is being used increasingly to investigate equine lameness at many centres, and

variation between clinicians in the interpretation of scans is an area of concern which requires considerable further attention if this method of imaging is to become a valid measurement tool.

There was no significant correlation between type of scan pattern and radiographic feature although it did seem that linear patterns were more common in the PIP and TMT joints and were almost significantly correlated with a higher osteophyte score. A larger study should be made to clarify this finding.

It was not possible to measure the sensitivity of the scoring system to detect change without a known outcome measure with which to compare it - however it may well be that the interval of 6 months between scans was not long enough to detect change once a joint had become scintigraphically "hot".

The use of scintigraphy was useful in detecting increased subchondral bone activity in three horses (5, 8, and 19) in which there were no radiological signs of OA despite the lameness in two of the cases (5 and 19) being of more than 18 months duration.

The lack of difference in sensitivity between scintigraphy and radiography for diagnosis of cases of metacarpophalangeal and tarsal OA has previously been reported (Lamb *et al.* 1989) but the prevalence of chronic cases of OA in which radiological signs of OA were already present could have accounted for this finding. However in that study, as in this one, scintigraphy was useful in some cases where radiographic changes were not present and was therefore considered a valuable additional diagnostic aid.

Scintimetry

The calculation of AI was found to be reproducible but did not show any significant change between the first and second scans for either treatment group. It was unexpected that there was no significant difference between the clinically active OA joint and the contralateral, but this may have been because of the larger number of cases in which the disease was bilaterally symmetrical.

There was a strong correlation between the scintigraphy score of uptake and the AI cross-sectionally but not longitudinally - this may have been because of the reasons mentioned above i.e. poor detection of change demonstrated by the scintigraphy scoring or inadequate duration of the trial.

An interesting finding was the positive correlation (although only just significant) between global improvement in lameness and the AI change of the clinically active joint. This finding implies that a clinical improvement correlates with an increase in subchondral activity. If this is a true finding it would be in opposition to the commonly held view that an increase in subchondral bone activity represented by increased uptake of isotope represents worsening of the OA condition. It is possible that the increase in isotope uptake in these cases is a reflection of adaptive bone remodelling.

The lack of difference between the normal reference bone in the lame and sound leg was unlike that reported previously (Attenburrow 1997) where the sound leg often has an increased uptake, understood to be because of increased weightbearing. In this study this may have been because of the high incidence of bilateral disease.

Summary

1. Radiographic scoring of JSN, osteophytes and global signs of OA was found to be reproducible cross-sectionally both intra- and inter-observer.

2. Scintigraphy was useful as an additional method of OA diagnosis when used in combination with clinical and radiographic methods.

3. The scintigraphy scoring system was only found to be reliable intra-observer.

4. The use of scintimetry was found to be reliable cross-sectionally and correlated well with scintigraphy scores but provided little additional information.

5. There was no correlation between radiography or scintigraphy changes and global change in lameness. Scintimetry was weakly positively correlated with global lameness change - this finding requires further evaluation.

6. No correlation could be detected between scintigraphy patterns and radiographic features of OA.

7. In this study a difference in outcome between the two treatment groups in the CaPPS trial has not been detected by radiology nor scintigraphy.

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Chapter Seven

Variation of biochemical markers in normal equine joints.

Introduction

During normal metabolism joint tissue macromolecules or fragments thereof are released into the synovial fluid, blood and urine. Biochemical or immunochemical assays can be used to detect these molecules. In osteoarthritis (OA), changes in metabolism can produce compositional changes in newly synthesised molecules and the resulting neoepitopes can be detected by immunochemical methods. The aim of molecular marker research is to use these molecules or epitopes as markers to detect early OA changes at a stage at which other diagnostic techniques may be ineffective, to monitor progression of the disease, and to prognosticate on the outcome. Although these aims have not yet been realised, investigation into these markers in itself is providing a wealth of information regarding the metabolism of the joint and the pathogenic mechanisms involved in the development of OA.

Marker interpretation

Cartilage matrix macromolecules initially undergo extracellular degradation, after which a proportion of these molecules or fragments are taken up by the chondrocytes and further degraded. Others reach the synovial fluid by diffusion. Here they may be degraded by the synovial cells or flow on into the lymph with the synovial fluid (Fraser *et al.* 1988). A large proportion of macromolecules are removed or at least further degraded by the lymph nodes. The remaining are removed from the circulation by liver cells, with exception of the collagen cross links, which are resistant to degradation and are finally eliminated in the urine.

The concentration of a cartilage or other joint tissue marker within the synovial fluid provides information only regarding the condition of the tissue within that joint, while joint marker measurement in serum is related to the whole body or all the joints in the body, depending on the origin of the marker. Although the ultimate aim would be to find a serum marker, because of the ease with which samples can be obtained, there are obviously several disadvantages to marker measurement in this compartment. A large proportion of macromolecules produced by the joint tissues will have been degraded by the time the circulation is reached, and those that

do arrive in the blood are diluted by similar markers from healthy joints. The concentration of markers is much lower than is found within the joint and this may cause problems in detection, since the circulating concentrations of some markers may be below the sensitivity of the assays.

The concentration of a marker in joint fluid or serum cannot be interpreted quantitatively until the kinetics of its production and clearance from the joint are known (Levick 1990). Although the lymphatics are the major clearance pathway for these macromolecules, the relative rate of phagocytosis by the synovial lining cells is not known. In the diseased joint it is not only the rate of marker production that is affected but also the rate of synovial fluid turnover, and these rates may vary between different joints and in different types of joint disease. Different markers may also have different clearance kinetics, since the permeability of the synovial membrane is not uniform to all macromolecules (Levick *et al.* 1995). Great caution should therefore be applied when interpreting data on cartilage markers (Wallis *et al.* 1987). In order to make some allowance for these unknown variables in many studies markers are expressed as ratios to other markers.

Marker validation

The "ideal" marker of cartilage degradation is one which is not found in normal cartilage or in any other tissue. It is also one which can be validated against another form of measurement, validity being defined as "the extent to which any instrument measures what it is supposed to measure" (Carmines *et al.* 1979). There are a number of different classifications of validity but criterion validity is considered the most applicable test for biological phenomena i.e. does the marker measure the ongoing biological activity it is supposed to measure? This must correlate with a gold standard such as radiography or magnetic resonance imaging (MRI).

A set of modified "Koch's postulates" for biochemical markers of the state of OA have been proposed (Felson 1995) :

1) A marker is biologically credible.
2) A marker is regularly (universally) found in patients with OA of the joint.
3) Normal values for marker by age are defined sensibly.
4) With change in the state of OA, markers change appropriately.

Many of the putative markers of OA are not markers of existence or non-existence of disease but rather markers of disease dynamics or severity. These are often classified as prognostic markers. Suggested criteria for validation of prognostic markers of OA are as follows :

1) There should be a strong biological rationale.
   - The marker is identifiable in cartilage or periarticular bone.
   - If the marker is a measure of OA dynamics, its role in the pathogenesis of OA must be at least partly understood.
   - If the marker is a measure of disease severity, it must correlate with disease severity as determined by imaging and/or functional assessment.
2) A marker measured at baseline in a body fluid predicts the course of OA. The course of OA at baseline and follow up is determined independently of marker measurement using conventional clinical means.
3) The marker must be validated in patients with spectrum of mild/severe disease and OA of different aetiologies (e.g. post-traumatic v primary).
4) Marker measurement is reliable (repeatable) and described in sufficient detail as to be replicable by others.

Another function of markers is as predictors of responsiveness to therapy. These markers should be assayed at baseline in a longitudinal study , and those patients who responded to treatment, defined independently of the marker, should have different marker levels at baseline than those who did not respond. Markers of response to therapy are different - here the difference between marker level before and after treatment is important .

Types of marker

A large number of potential markers of joint tissue turnover have been identified - as well as the macromolecules produced by cartilage and bone turnover, these include the cytokines, enzymes and their inhibitors involved in joint metabolism. The choice of marker depends on the development of sensitive and specific assays with which to measure them. Markers have been classified in many different ways but here they will be described under their currently understood function.

1. Markers of cartilage matrix turnover

a) Cartilage matrix macromolecules

Proteoglycans:

Proteoglycan (PG) and PG fragments released into the SF during normal cartilage matrix turnover can be measured by ELISA. These substances have been found to be increased in human knee joints in post traumatic injuries and early OA (Lohmander *et al.* 1989; Ratcliffe *et al.* 1988). In dogs with experimentally induced OA the SF PG concentration was found to be increased (Heinegard *et al.* 1985) while in the horse the PG level in carpal and metacarpophalangeal joints was increased in chronic OA (Alwan *et al.* 1991).

Total glycosaminoglycans:

The level of total glycosaminoglycans (GAG) in the synovial fluid and serum have been measured, usually by the direct dye binding method (Farndale *et al.* 1986) in many studies and are thought to reflect cartilage destruction in OA (Alwan *et al.* 1991). Increased levels have been found in the SF, serum and urine of horses with OA, in the SF but not in the serum of dogs (Arican *et al.* 1994).

More specific studies have been made of individual glycosaminoglycans by more sensitive methods, in particular immunoassays. Caution must be employed when comparing results of these assays from different laboratories since differences in the epitopes measured are common.

Keratan sulphate (5D4):

Keratan sulphate (KS) is a sulphated GAG which is covalently attached to the protein core of the proteoglycan molecules. Some KS chains have a highly antigenic oversulphated epitope, which is specifically recognised by the monoclonal antibody (1/20/5D4) used in this study (Caterson *et al.* 1983). The significance of the levels of this KS epitope in the synovial fluid and the serum and the changes related to joint disease have been studied widely (Campion *et al.* 1991; Sweet *et al.* 1988; Thonar *et al.* 1992; Todhunter *et al.* 1993). Traditionally it was believed that KS was a marker of cartilage degradation since serum levels were found to be higher in OA cases compared to normal controls (Sweet *et al.* 1988; Thonar *et al.* 1992). However, since more recent studies, the more broad description of KS as a marker of cartilage turnover has been used. In a study by Todhunter (Todhunter *et al.* 1997) , KS 5D4 concentrations, measured by ELISA, were found to be lower in OA joints compared to

normal controls. Breed, gender, and type of other joint disease were found to affect the marker concentration however.

b) Noncollagenous proteins

Cartilage oligomeric matrix protein:

Cartilage oligomeric matrix protein (COMP) consists of five 100 kDa subunits and was first isolated from bovine articular cartilage (Hedbom *et al.* 1992). It is found throughout all types of cartilage and also in non cartilaginous tissues e.g. tendon (Smith 1997). Little is known about its specific physiological function although it has been shown to bind chondrocytes (Dicesare *et al.* 1994) and could play a role in contacts between extracellular matrix and chondrocytes. It is thought to be a marker of cartilage matrix turnover.

Studies have been carried out investigating this marker in SF and serum in four types of arthritis in man, using immunoassays, and in all four diseases COMP was higher in SF than serum, suggesting local production of this protein in joints (Saxne *et al.* 1992) . High COMP levels have also been found in patients shortly after knee injury (Lohmander *et al.* 1994). More recently a study has demonstrated serum COMP increased significantly in the first year of follow up only in those patients whose knee OA progressed (Sharif *et al.* 1995), and another reports that serum concentrations of COMP correlated strongly with scintigraphic scores in OA (Petersson *et al.* 1995). Nothing could be found in the literature regarding COMP in equine OA, or concerning possible variation in synovial fluid COMP from normal joints.

***Fibronectin*:**

Fibronectin is a small protein synthesised and accumulated in OA joints in increased amounts (Lust *et al.* 1987) .

***YKL-40*:**

This is a 40kDa protein secreted by human articular cartilage cells and synoviocytes and is present in increased concentrations in OA (Johansen *et al.* 1993).

c) Enzymes

Metalloproteinases:

The levels of metalloproteinases (MMPs) in the SF can be used as markers of cartilage degradation (Lohmander *et al.* 1993). These enzymes are produced by the synovium and articular cartilage. In man it has been found that concentrations of stromelysin (MMP-3) in

knee SF were raised for many years following knee injury (Lohmander *et al.* 1993). The ratio of MMP-3 and tissue inhibitor of metalloproteinases (TIMP) was also found to change from an excess of MMP-3 in the injured joint to an excess of TIMP in the normal reference joint (Lohmander *et al.* 1994). In an equine study, the enzyme activity of the gelatinases MMP-2 and MMP-9 was measured by gelatin zymography and was found to be increased in synovial fluids in septic and non-septic joint disease compared to normal controls (Clegg *et al.* 1997).

Aggrecanase:

Cartilage aggrecan catabolism by the as yet uncharacterised enzyme "Aggrecanase" generates neoepitopes which can be detected by monoclonal antibodies (Hughes *et al.* 1992). BC-3 and BC-4 are monoclonal antibodies that recognise the N terminal in the interglobular domain of the aggrecan molecule, generated by the unknown enzyme Aggrecanase and the new C terminal in the interglobular domain (IGD) generated by the MMPs.

d) Collagen

Monoclonal antibodies are now available to measure the level of type II collagen synthesis and degradation by detection of an intra-α-chain epitope (Poole *et al.* 1995) in the helical region of the denatured molecule. These antibodies show no reactivity with the intact triple helical type II collagen (Hollander *et al.* 1994).

2. Markers of cartilage synthesis

Chondroitin sulphate:

The monoclonal antibodies 3B3 and 7D4 can be used to identify subtle biochemical differences on newly synthesised chondroitin sulphate chains in OA cartilage. 3B3(+) recognises as its epitope a non reducing terminal unsaturated uronic acid residue adjacent to N-acetylgalactosamine-6-sulphate after the CS chains have been digested with chondroitinase. 3B3(-) recognises a mimotope containing a saturated glucoronic acid residue at the nonreducing terminal that occurs in CS chains in osteoarthritic cartilage. Proteoglycans containing the 3B3(-) epitope occur only infrequently in normal cartilage (Caterson *et al.* 1990). Another epitope, for antibody 7D4, is less well recognised, but is also more prevalent on OA cartilage (Slater *et al.* 1995). The 846 antibody recognises a native epitope that also has increased expression in proteoglycans from OA cartilage (Rizkalla *et al.* 1992).

Expression of these neoepitopes seems to reflect a switch in the articular cartilage phenotype as a result of the tissues attempt to repair. These markers have been investigated in body fluids in animals and man (Poole *et al.* 1994; Ratcliffe *et al.* 1996; Ratcliffe *et al.* 1993). Concentrations in SF are considerably higher than those in serum, and SF levels correlate inversely with the levels of KS (Poole *et al.* 1994).

A change in the sulphation pattern of chondroitin sulphate in the cartilage matrix can also indicate early OA changes. The ratio of chondroitin 6-sulphate (C6) to chondroitin 4-sulphate (C4) decreases as more of the immature isomer C4 is produced (Sharif *et al.* 1996; Shinmei *et al.* 1992).

Collagen:

The C-propeptide (PIIP) of type II collagen has been investigated as a marker of collagen biosynthesis (Shinmei *et al.* 1993). It is present in joint fluids in osteoarthritis and although these levels are not very elevated over serum levels, they may reflect local cartilage changes (Poole *et al.* 1995). Inverse changes in levels of PIIP and KS have been noticed which may suggest the existence of phases of net cartilage synthesis and degradation (Poole *et al.* 1995).

3. Markers of bone turnover

a) Bone formation

Bone specific alkaline phosphatase:

Significant improvements have recently been made in the measurement of alkaline phosphatase (AP), which has been used as a marker of bone formation for many years. Serum total alkaline phosphatase activity is the most commonly used marker of bone formation (Delmas 1995). However several tissues contain alkaline phosphatase, liver and bone isoenzymes being the major contributors to the serum levels, although the intestine, and placenta in the pregnant animal are also contributors. For these reasons total AP lacks specificity and sensitivity as a marker. Recently a monoclonal antibody that recognises preferentially the bone isoenzyme (BAP) (Hill *et al.* 1986) has been developed.

BAP is located on the cell surface of osteoblasts and there is evidence to suggest that BAP plays an important role in bone mineralisation (Yoon et al 1989, Brixen et al 1989, Caswell 1991). In man it has been shown that its activity increases with ageing in adults, and especially in women after the menopause (Crilly *et al.* 1980). BAP has been measured in

serum and synovial fluid in patients suffering from both OA and rheumatoid arthritis (Sharif *et al.* 1995) and although no normal controls were included, SF BAP levels were found to be lower than those in serum, and the BAP concentration in OA SF was lower than that in RA SF. There does not appear to be a diurnal rhythm for serum BAP in man (Cromier 1995). No studies could be found in the literature concerning the measurement of BAP in equine synovial fluid but equine serum BAP has been reported to decrease with age particularly during the first 2 years (Price *et al.* 1995).

Many different methods have been used to measure BAP in horse sera, including the wheat germ precipitation test (Hank *et al.* 1993) and the immunoradiometric assay (Tandem -Ostase Hybritech Belgium) (Jackson *et al.* 1996). As yet there are no published data concerning the use of the assay employed in this study (Alkphase-B Metra Biosystems) on equine samples. Investigations into the use of this assay with human serum samples have shown (Gomez *et al.* 1995) no cross reactivity occurring with intestinal or placental alkaline phosphates, and only 3 -8% cross reactivity to the liver isoenzyme. The assay has been used successfully in canine studies (Innes 1997) in both serum and synovial fluid in which BAP activity was found to be increased in clinically active OA joints .

Osteocalcin :

Osteocalcin (OC) is a small bone specific noncollagenous protein synthesised by osteoblasts with a high affinity for hydroxyapatite, and recent results have shown a positive correlation between serum OC and scintigraphical bone scans (Sharif *et al.* 1995). A diurnal rhythm has been demonstrated in man (Gundberg *et al.* 1985) and in the horse (Lepage *et al.* 1991).

Bone sialoprotein :

Bone sialoprotein (BSP) is a bone specific molecule produced by osteoblasts, and constitutes 12 % of the noncollagenous proteins of bone matrix. Its function is unknown but it binds to hydroxyapatite. It shows the highest level of synthesis at the cartilage bone interface of growth cartilage and is markedly enriched at the cartilage bone interface, the tidemark and in mineralising osteoid (Debri *et al.* 1995; Hultenby *et al.* 1994; Riminucci *et al.* 1995). Significant increases in the SF concentration of BSP have been reported in connection with acute joint trauma (Saxne *et al.* 1995) and there is a positive association with the degree of joint damage in RA patients (Saxne *et al.* 1995). BSP levels increase over time in RA patients with progressive joint disease (Mansson *et al.* 1997). In OA an increased level is thought to

be associated with remodelling of the cartilage bone interface and subchondral bone (Lohmander *et al.* 1996).

b) Bone resorption

Collagen crosslinks:

The collagen crosslink deoxypyridinoline is primarily found in bone and is a more specific marker of bone resorption than pyridinoline which is also present in cartilage and other connective tissues. The levels of these markers have been found to be raised in the urine of OA patients (Seibel *et al.* 1989) and correlate positively with the Kellgren and Lawrence radiography scale (Thompson *et al.* 1992).

4. Markers of synovial activity

Hyaluronan:

Hyaluronan (HA) is a GAG produced by the synovial lining cells, and by chondrocytes in cartilage, having important roles in the mechanical properties of the synovial fluid and, as the backbone to proteoglycan aggregates, in the compressive stiffness of the cartilage. It differs from other GAGs in that it is a linear polysaccharide, lacking sulphated side chains and not being covalently linked to protein. It is a very large molecule (M_r 10^4kD) with rheological properties showing both viscoelasticity and shear dependence which are connected with its lubricating properties in the joint. Although HA is synthesised by fibroblasts in several tissues it is found in highest concentration in the synovial fluid (SF). It passes through the synovial membrane to the lymphatics and then in part to the circulation from where it is cleared by the endothelial cells of the liver and kidney (Woessner 1991).

The use of HA as a biochemical marker of osteoarthritis has been investigated in man and horse in serum and synovial fluid. Specifically it is reported to be a marker of synovial membrane activity. It has been shown in man that there is a diurnal rhythm of serum HA (Engstrom-Laurent *et al.* 1987), but no such information exists for the horse. The concentration of HA has been found to be significantly lower than that in normal controls in osteoarthritic equine carpal joints (Hilbert *et al.* 1984; Tulamo *et al.* 1994) and in man (Hedin *et al.* 1991). HA in equine serum is positively related to age (Tulamo *et al.* 1990) and in man serum HA is significantly increased in OA cases (Hedin *et al.* 1991) and correlates with both degree of cartilage damage and disease duration (Sharif *et al.* 1995) . Serum HA has also been found to be a predictor of disease progression in OA of the knee (Sharif *et al.*

1995) . While it has been noted that there is a wide variation in SF and serum values of HA in the horse (Saari *et al.* 1989) the potential difference in SF HA between different normal joints has not been reported in the literature. This information is essential for meaningful interpretation of HA values.

Having reviewed the available literature it is clear that only a limited amount of data on reference concentrations and longitudinal and cross sectional marker variations has been published, and there is a need for more information on reference ranges of these markers in the normal population, along with the effect of variables on these values, i.e. age, gender, disease duration, exercise and joints affected. The future of markers as outcome measures in clinical trials of new treatments for OA will require the general availability of reproducible assays and data on the "normal" or base line range of concentrations of these markers in reference populations (Lohmander *et al.* 1997).

The investigation into normal variation, which was the objective of the present study, can also provide information about the metabolism of cartilage within different joints.

In this study it was decided to investigate the markers KS, GAG, and COMP as markers of cartilage matrix turnover, BAP as a marker of bone formation, and HA as a marker of synovial activity.

The aims were as follows:

Aims

1. To establish a normal range of values of KS, GAG, HA, COMP and BAP in equine synovial fluid, and to investigate whether there was any variation in levels between different normal joints .

2. To establish the variation in the normal levels of these markers with age.

3. To establish the normal range of values of HA in equine serum.

4. To establish whether diurnal rhythm exists for equine serum HA.

Methods

Horse and sample collection

Synovial fluid samples were taken, immediately following slaughter, by needle aspiration from the joints of 53 horses which had been destroyed for reasons unrelated to locomotor system disorders. The total volume possible to remove and the viscosity of the SF were noted in each case. The samples were taken from the distal interphalangeal (DIP), proximal interphalangeal (PIP), metacarpophalangeal (MCP), metatarsophalangeal (MTP), tarsometatarsal (TMT), tarsocrural (TC), femoropatellar (FP), and antebrachiocarpal (ABC) joints. In a subgroup of 11 horses values for KS , GAG, COMP and HA were obtained for the distal interphalangeal, proximal interphalangeal, and metacarpophalangeal /metatarsophalangeal from the same limb in each animal. Following sampling each joint was dissected open and examined visually for any abnormality. Any joint showing signs of osteoarthritis or any other joint disease was excluded from this study.

The age of each horse was assessed by an examination of the eruption and table appearances of the teeth. In some cases ages were verified by available passports. Horses were assigned to a group according to age, i.e. 5-10 years , 10 - 15 years etc. This was to allow for recently recognised inaccuracies in the technique of ageing horses by examination of the teeth (Richardson *et al.* 1995). The synovial fluid samples were centrifuged at 4000rpm for 10 minutes to remove cells and debris. The supernatant was then aliquotted and stored at -20° C initially, and then -80°C until analysed.

Materials

96 well polystyrene assay plate	-	Nunc (Immuno Plate Maxisorp), Life Technologies, Paisley, UK
Chondroitinase ABC treated porcine proteoglycan	-	Prepared in lab by Miss K. Meadows
Human proteoglycan AIDI	-	Prepared in lab by K. Meadows
5D4 monoclonal antibody	-	Provided by Professor Bruce Caterson
Hyaluronic acid	-	Sigma Chemicals, Poole, Dorset, UK
Bovine tracheal chondroitin sulphate A	-	Sigma Chemicals
Dimethylene Blue dye	-	Serva, Heidelberg, FRG
Buffers		
NaC03	-	Sigma Chemicals
NaHC03	-	Sigma Chemicals
NaCl	-	Sigma Chemicals
Na2 HP04	-	Sigma Chemicals
KH2P04	-	Sigma Chemicals
Sodium Azide	-	BDH, Merck Ltd, Poole
Bovine serum albumin (BSA)	-	Sigma Chemicals
Tween 20	-	BDH, Merck Ltd
Alkaline phosphatase conjugated antimouse IgG	-	Sigma Chemicals
Alkaline phosphatase substrate tablets	-	Sigma Chemicals
EDTA	-	Sigma Chemicals
Papain	-	Sigma Chemicals
Glycine	-	Sigma Chemicals
BAP assay kit	-	Alkphase-B, Metra Biosystems Inc., CA, USA
Automated plate reader	-	Labsystems Multiskan, Life Sciences International

Keratan Sulphate Assay

Samples were analysed by ELISA inhibition using a method similar to that described by Thonar *et al* 1985 but with a few minor modifications (Creamer *et al.* 1994) (Figure 7-1). The antibody used was a mouse IgG monoclonal 5D4 (Caterson *et al.* 1988) which recognises a sulphated epitope on certain keratan sulphate chains. The standard antigen was chondroitinase ABC treated porcine proteoglycan. The 96 well assay plate was coated with 200µl per well of 48ng/ml PG antigen, diluted 1:10,000 in 50mM Carbonate/Bicarbonate buffer at pH 9.6. This was incubated for two hours at room temperature and then overnight at 4°C. On a separate dilution plate, porcine proteoglycan antigen was added in duplicate in double dilutions to produce a standard curve in the range 200ng/ml - 1.9 ng/ml. Phosphate buffered saline was used for dilutions with 0.02% Sodium Azide, 1% BSA and 0.05% Tween 20 added at pH 5.3. 125µl of each sample, appropriately diluted in this buffer (dilution ranged from 1:1000 to 1: 8000) were added in duplicate to the plate. A control sample of known value was used in each assay. Two blank wells consisting of buffer only and two inhibition wells consisting of antibody and buffer only were included. 125µl of 5D4 Antibody, diluted 1: 25000, was added to each well before incubation for 1 hour at room temperature and then overnight at 4°C. On the second day of the assay, the antigen-antibody complex was transferred to the coated plates. 200µl of conjugated alkaline phosphatase anti-mouse IgG (Sigma A-2179) diluted 1:2500 was added to each well and incubated at room temperature for 1 hour. After washing the plates thoroughly in phosphate buffered saline, 200µl alkaline phosphate substrate solution (Sigma 104) was added to each well. The absorbance of each well was read after a further incubation of 30 minutes, at 405nm.

The concentration of keratan sulphate in the synovial fluid samples was calculated using only the straight portion of the standard curve and all samples were assayed in duplicate. Interassay coefficient of variation was calculated using both the 50% inhibition point of each assay, and the value of the control sample, and this proved to be < 10%. The intra-assay variation was < 5%.

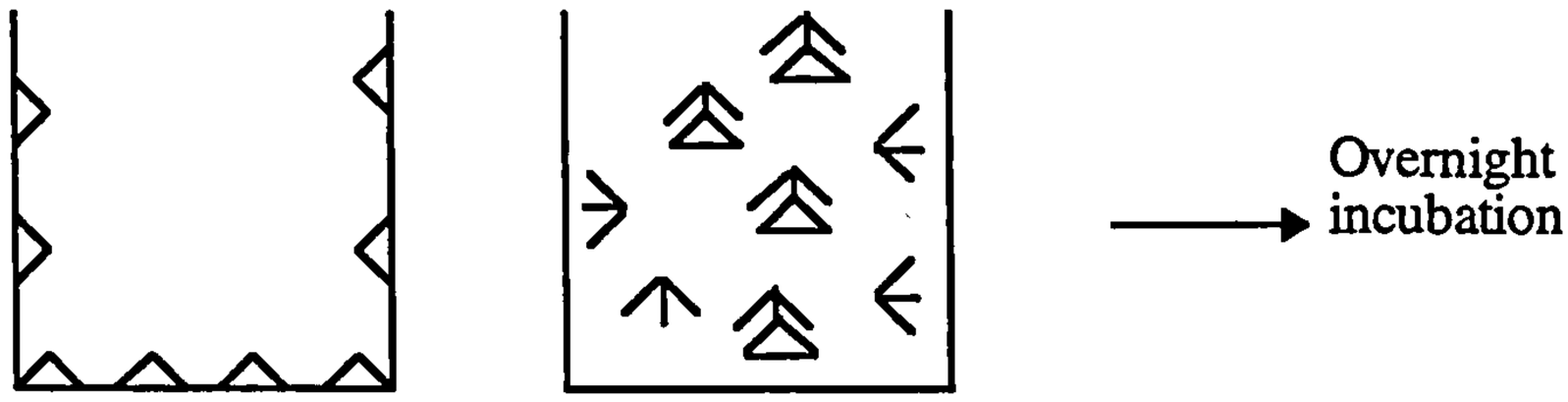

Figure 7-1: Diagram describing process of KS(5D4) ELISA

Total Glycosaminoglycans Assay

GAG levels were measured by dimethylmethylene blue assay described by Farndale (Farndale *et al.* 1986) and using a standard bovine trachea chondroitin sulphate for calibration. 50µl of synovial fluid, diluted if necessary up to 1:4 with disodium hydrogen phosphate buffer, were mixed with 50µl papain which had been diluted to 32.6 U/ml in the same buffer. The samples were incubated in stoppered tubes for 2 hours in a 65°C waterbath to allow protein digestion to occur. A standard bovine trachea chondroitin sulphate was used for calibration and this and the digested samples were added in 20µl aliquots in duplicate to a 96 well ELISA plate. Buffer was used for blanks. Dimethylene Blue buffer was prepared by mixing 16mg Dimethylene Blue (DMB) (Serva 20335), 3.04g Glycine and 2.37g NaCl in 1L distilled water. The pH was adjusted to 3.0 with concentrated HCl. 200µl of this DMB buffer were added to all wells on the ELISA plate and the absorbance at 525nm and 690nm wavelengths was read immediately in a spectrophotometer.

All samples were assayed in duplicate. The interassay and intra-assay coefficient of variation were < 7% and < 12% respectively.

Cartilage oligomeric matrix protein assay

COMP[1] concentrations in the synovial fluids were measured using an inhibition ELISA and a polyclonal anti equine COMP (etCOMP) antibody (Smith 1997). 200µl of equine tendon purified etCOMP was added to each flat bottomed well of a 96-well Maxisorp ELISA plate (Nunc). Coating was achieved by overnight incubation at room temperature. On a second plate (Sterilin 96 well plate (Labsystems)) a standard curve using 115µl of the standard etCOMP purified from equine tendon with concentrations of 0.2µg/ml to 0.00625µg/ml was set up . Appropriately diluted synovial fluid samples were added in 115µl aliquots to remaining wells. 115µl of anti etCOMP antibody was then added to all wells. All standards and samples were assayed in triplicate. After 1 hour incubation at room temperature, 200µl of resulting well contents was transferred to the previously coated plate. 200µl alkaline phosphatase conjugated goat antirabbit IgG (Orion Diagnostica) was then added to each well. Following a further 1 hour incubation 200µl of the substrate p-nitrophenyl phosphate (Sigma 104 phosphatase substrate -Sigma Chemical Co) was added before reading the absorption of each well immediately at 405nm on a Labsystems Multiscan plate reader.

Bone specific alkaline phosphatase assay

This was carried out using a capture immunoassay available as a commercial kit (Alkphase -B; Metra Biosystems). The assay uses a monoclonal purified murine anti BAP IgG antibody coated onto a microtitre stripwells to capture BAP in the added sample. The enzyme activity of the captured BAP is detected by a p-Nitrophenyl phosphate (pNPP) substrate. BAP standards used are purified from osteosarcoma SAOS -2 cells in a buffered solution. High and low controls of known value were provided. 125µl of assay buffer containing magnesium chloride and zinc sulphate was added to each antibody coated well. Following this, 20µl of standard, control or synovial fluid sample were added and mixed by gentle swirling. After an incubation period of 30 minutes at room temperature the strips were emptied and wells washed with a non-ionic detergent buffer. 150µl of pNPP substrate solution was then added to each well before further incubation of 30 minutes at room temperature. After adding 100µl of NaOH stop solution the optical density of each well was read at 405nm using an automatic plate reader. Quantitation software (Deltasoft) with a quadratic curve fitting equation was used on a Macintosh LC computer to interpret values of unknowns from a linear standard curve.

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[1] This assay was carried out by Dr Roger Smith, Royal Veterinary College.

Hyaluronan assay

This inhibition ELISA assay was adapted from a method described by Goldberg (Goldberg 1988). 96 well polystyrene plates (outermost wells were excluded) were coated with 200µl of hyaluronan (Sigma) in a 20mM Sodium Carbonate buffer. The plates were incubated overnight at 4°C before blocking with 1% bovine serum albumin (BSA)-PBS, and further six hour incubation at 4°C. Before use plates were washed twice with 0.05% Tween -PBS. HA was diluted in 0.05% Tween - PBS/1% BSA and double diluted to make a standard curve ranging from 2000ng/ml to 0.977ng/ml. Samples were diluted (serum 1: 4 to 1: 8) and 100µl added in duplicate to the wells. PBS buffer was used as a blank, and a control sample and an inhibition well were added to check assay variation. 100µl of 1µg/ml Human PG was added to all wells except the blanks. After shaking for 20 seconds the plates were incubated overnight at 4°C. The plates were washed four times with Tween -PBS before adding 200µl 5D4 antibody (ICN) to all wells and incubating at 37°C for an hour. After four further washes in Tween -PBS 200µl of anti-mouse IgG alkaline phosphatase conjugate (Sigma A-2179) in Tween - PBS/BSA was added to each well. Plates were washed four times in Tween -PBS and then 200µl of Alkaline phosphatase substrate was added to each well. After allowing 30 minutes at room temperature for development of colour the colour absorbance of the plates were read on an automatic ELISA plate reader at 405nm. The interassay and intraassay coefficients of variation for SF were 22% and 11% respectively and for serum were 12% and 7.2%.

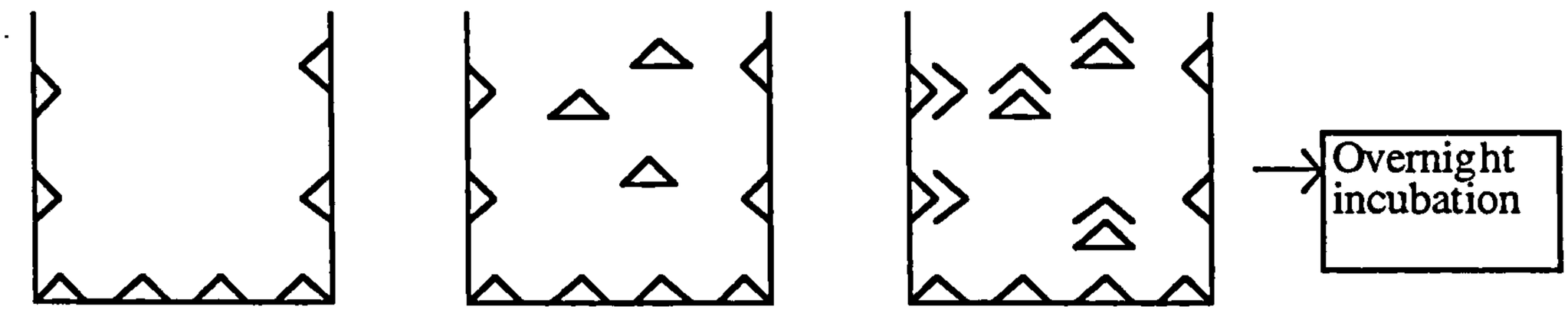

Figure 7-2: Diagram describing process of Hyaluronan inhibition ELISA.

Diurnal rhythm.

Six horses, two male and four female, aged between 4 and 20 years , were used for this study. None of the horses showed any clinical signs of musculoskeletal disease and each horse had haematological and biochemical profiles within normal limits for our laboratory. They were all stabled throughout the study period. Blood was sampled via indwelling jugular catheters at two hourly intervals over a 24 hour period. After centrifuging for 5 minutes at 3000rpm, the serum was removed and stored at -80C until analysis of HA concentration by ELISA.

Statistical Analysis

Inter joint variation :

Statistical analysis was by the Wilcoxon matched pairs test on matched samples i.e. those samples taken from joints in the same horse. The distribution of all values were skewed despite logarithmic transformation so the median (+ semi interquartile ranges) were chosen to demonstrate results. When joints from more than 1 leg in a horse were sampled, the results from only 1 leg, chosen at random, were used. Analysis of correlations were made by the Spearmans correlation coefficient (r_s).

Diurnal rhythm

The potential effect of diurnal rhythm on HA serum concentrations was analysed by the use of z scores (Z), where

Z = (value of serum HA at one time point - mean serum HA all time points) / standard deviation (SD)

This value provides a degree of fluctuation of serum HA throughout the day, relative to each horse. Logarithmic transformations were used to reduce the amount of variation in concentration. The Friedman repeated measures test were used to compare scores.

Results

Horse Number	Volume MCP (mls)	Volume PIP (mls)	Volume DIP (mls)
2	2.5	0.5	0.3
3	1.7	0.3	0.3
4	2.5	1	0.5
5	3	0.5	0.3
17	3.5	1	1
18	4	1	2.5
20	1.5	0.75	1
21	4	1.5	1.5
25	5	1	1
26	4	1.5	2
46	2	2	1

Table 7-1: Volumes of synovial fluid (mls) removed from each joint in matched group

Keratan sulphate and total glycosaminoglycans study

The mean age of horses in the study was 17 years but the sexes of the horses were not known. Results are expressed as KS (µg/ml), GAG (µg/ml), and KS:GAG ratio to allow for effects of synovial fluid volume. Values for equivalent joints from fore and hind legs were found to have the same median value and therefore were grouped together for analysis.

There was good correlation between the KS and GAG values for each joint sampled e.g. for the metacarpophalangeal joints r = 0.62.

Results of total samples

The results for KS, GAG and the KS:GAG ratio for all joints sampled are shown in Table 7-2 and the variation of KS is shown in Figure 7- 3.

It is clear from these results that there were differences in the median value of KS and GAG between different joints. The median ratio between KS epitope and total GAG remained relatively constant in the metacarpophalangeal/metatarsophalangeal, tarsometatarsal, tarsocrural, femoropatellar, and antebrachiocarpal joints but it increased in the proximal interphalangeal and distal interphalangeal joints.

Joint	n	KS µg/ml	GAG µg/ml	Ratio KS/GAG
Metacarpo/tarsophalangeal	60	20.6 (6.75)	79.7 (13.5)	0.25 (0.05)
Proximal interphalangeal	42	53.6 (20.7)	151 (35.8)	0.35 (0.08)
Distal Interphalangeal	25	144 (28.4)	268 (81.8)	0.47 (0.09)
Tarsometatarsal	23	12.6 (7.4)	57.9 (30.4)	0.22 (0.07)
Tarsocrural	8	24.9 (21.3)	142 (31.8)	0.24 (0.08)
Femoropatellar	9	55.9 (25.6)	210 (63.3)	0.29 (0.04)
Antebrachiocarpal	11	33.7 (10.3)	104 (30.3)	0.22 (0.08)

Table 7-2: Median values plus semi interquartile ranges for all joints sampled

KS = keratan sulphate (5D4 epitope), GAG = total glycosaminoglycans.

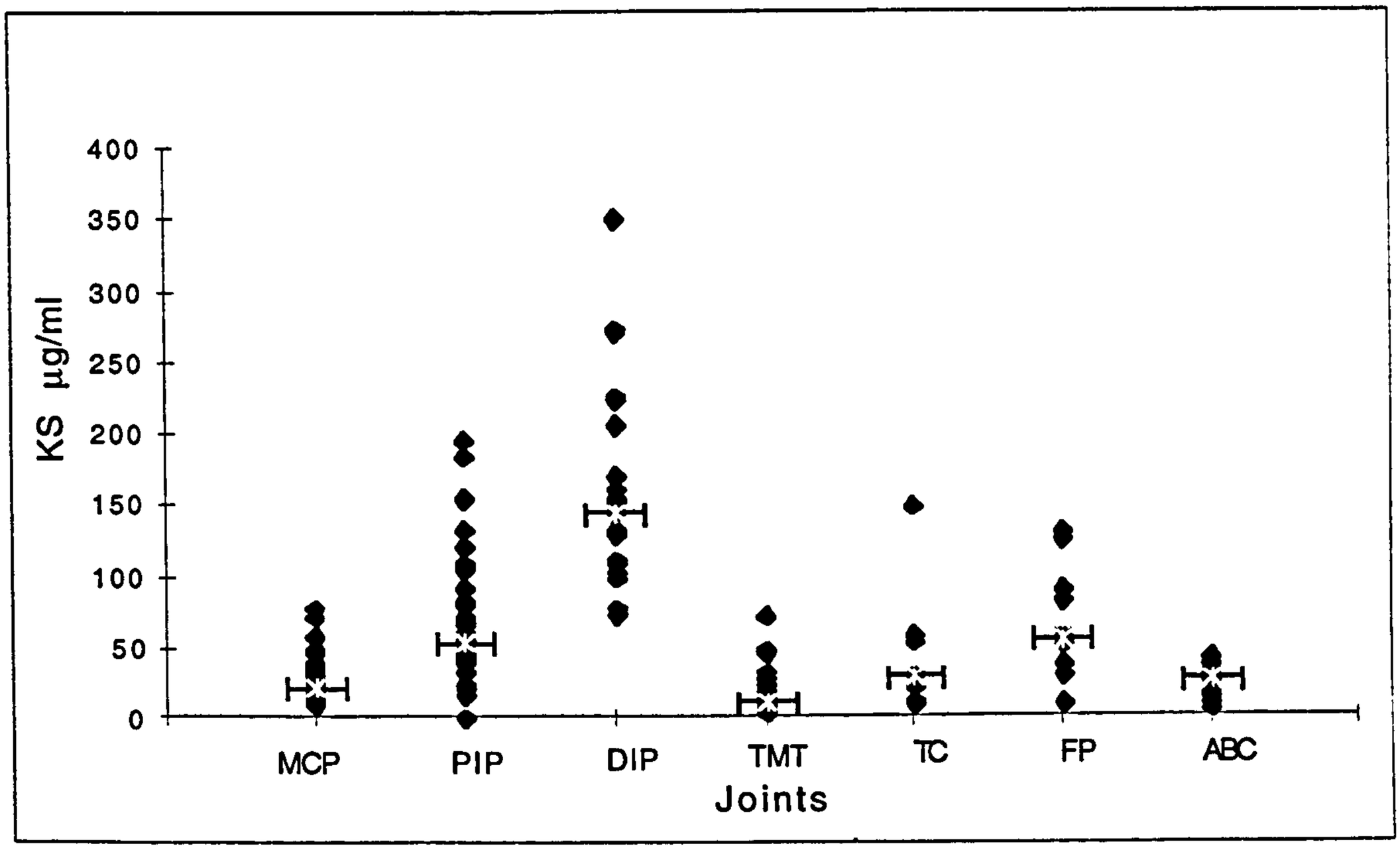

Figure 7-3: Variation of KS (5D4) plus median value in all joints

Key: MCP = metacarpophalangeal, PIP = proximal interphalangeal, DIP = distal interphalangeal, TMT = tarsometatarsal, TC = tarsocrural, FP = femoropatellar, ABC = antebrachiocarpal joint.

Variation between metacarpophalangeal, proximal interphalangeal and distal interphalangeal joints.

The variation between metacarpophalangeal, proximal interphalangeal and distal interphalangeal joints was investigated in a group of 11 horses, mean age 17.2 years, in which each of these three joints had been sampled in each horse. The results are shown in Figures 7-4a and b, and from these it can be seen that there were highly significant differences between KS and GAG levels and the KS:GAG ratio for each of the 3 joints with all three parameters increasing in median value from metacarpophalangeal to proximal interphalangeal and still further to distal interphalangeal (Table 7-2).

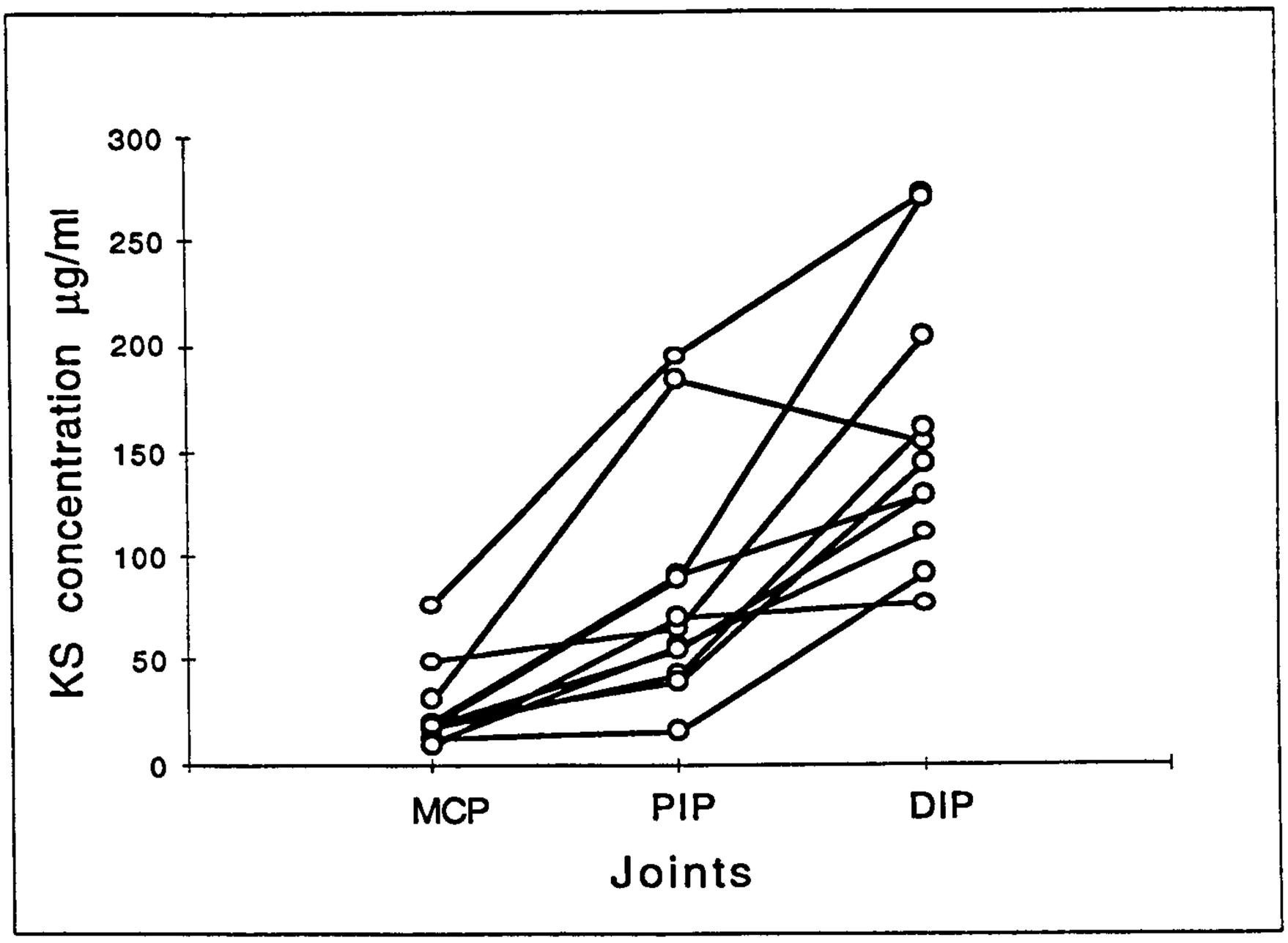

Figure 7-4a: Variation in KS SF concentration between normal matched joints

For abbreviations see Figure 7-3

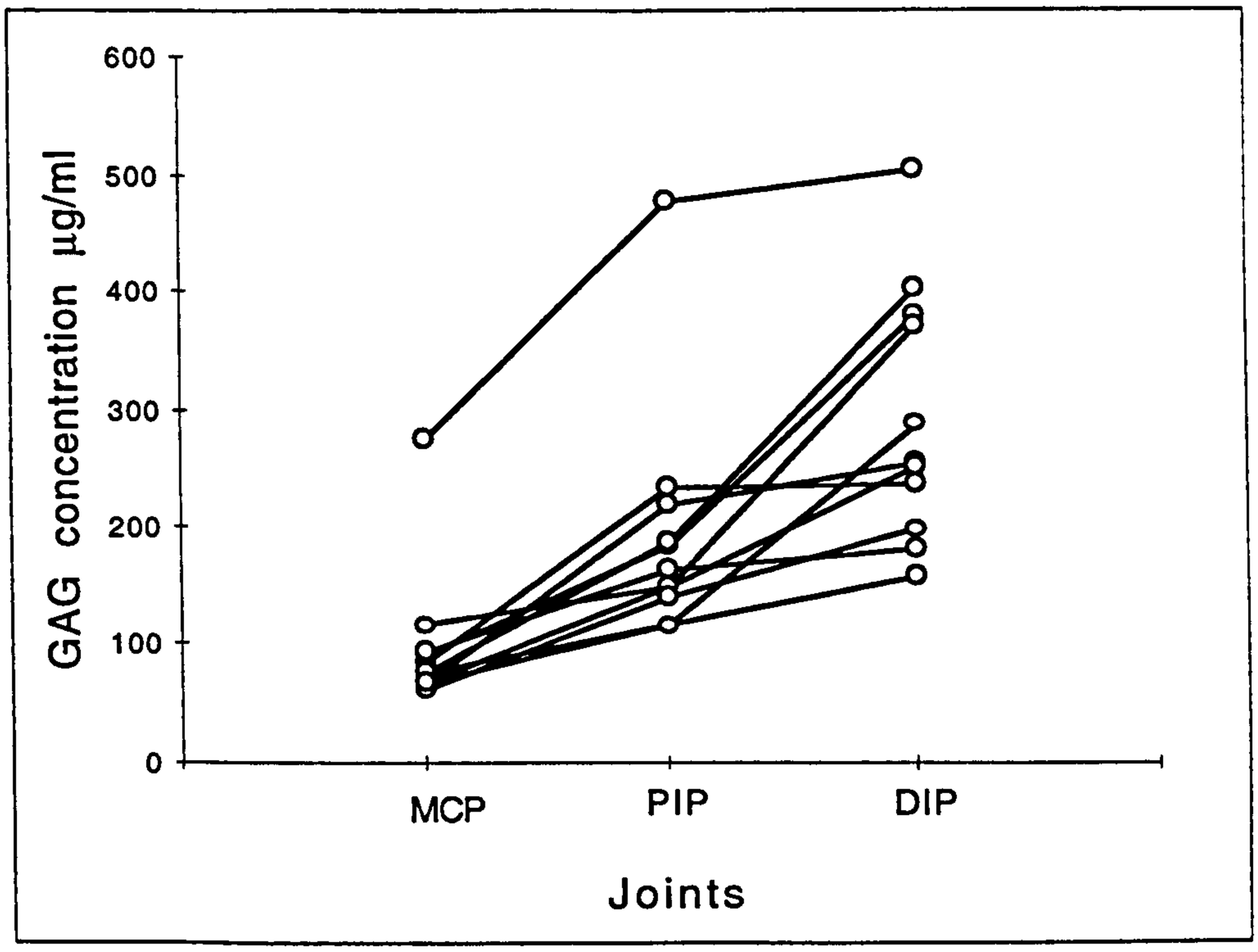

Figure 7-4b: Variation in GAG SF concentration between normal matched joints

For abbreviations see Figure 7-3

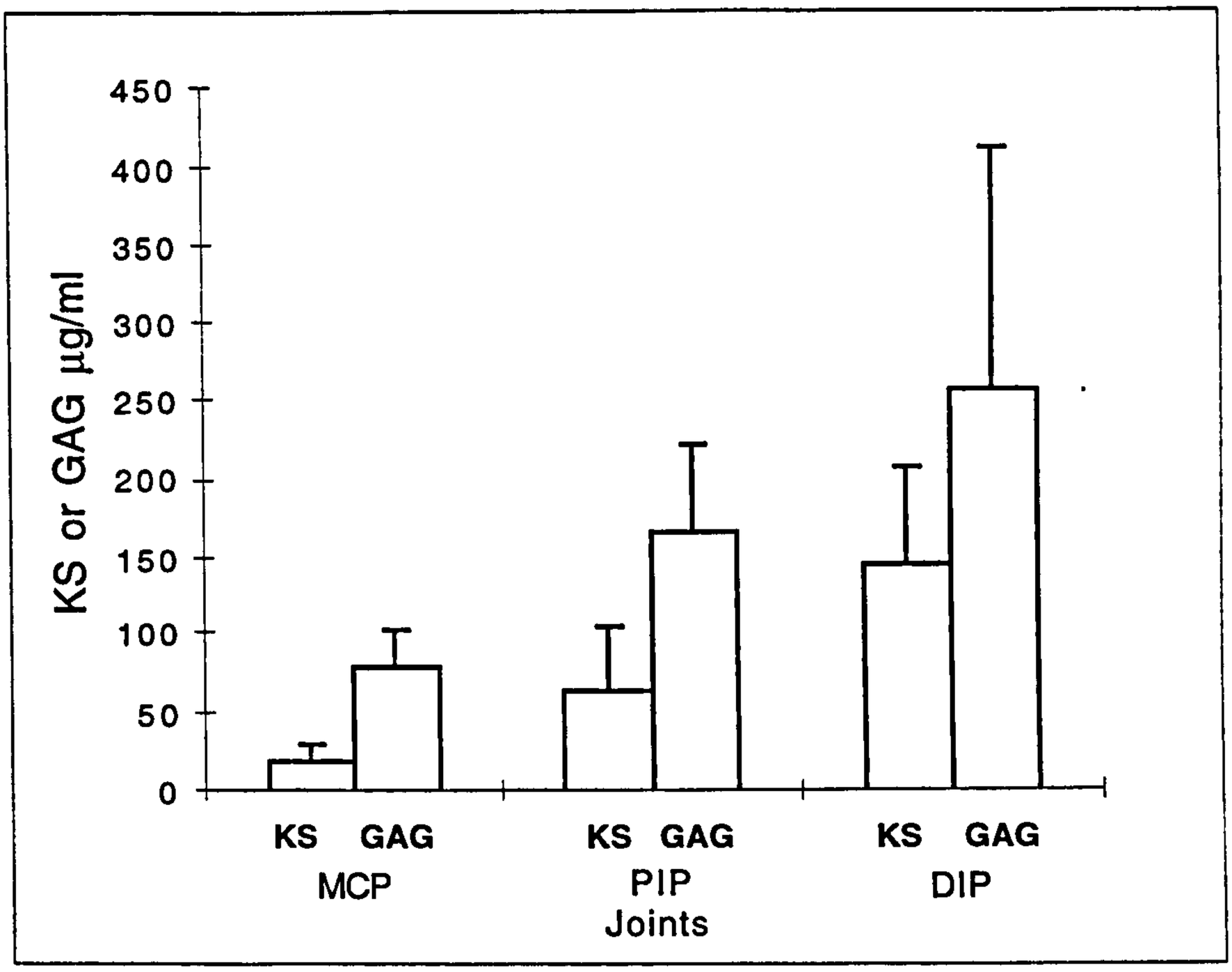

Figure 7-4c: Median KS and GAG plus semi-interquartile range in normal matched joints.

For abbreviations see Figure 7-3

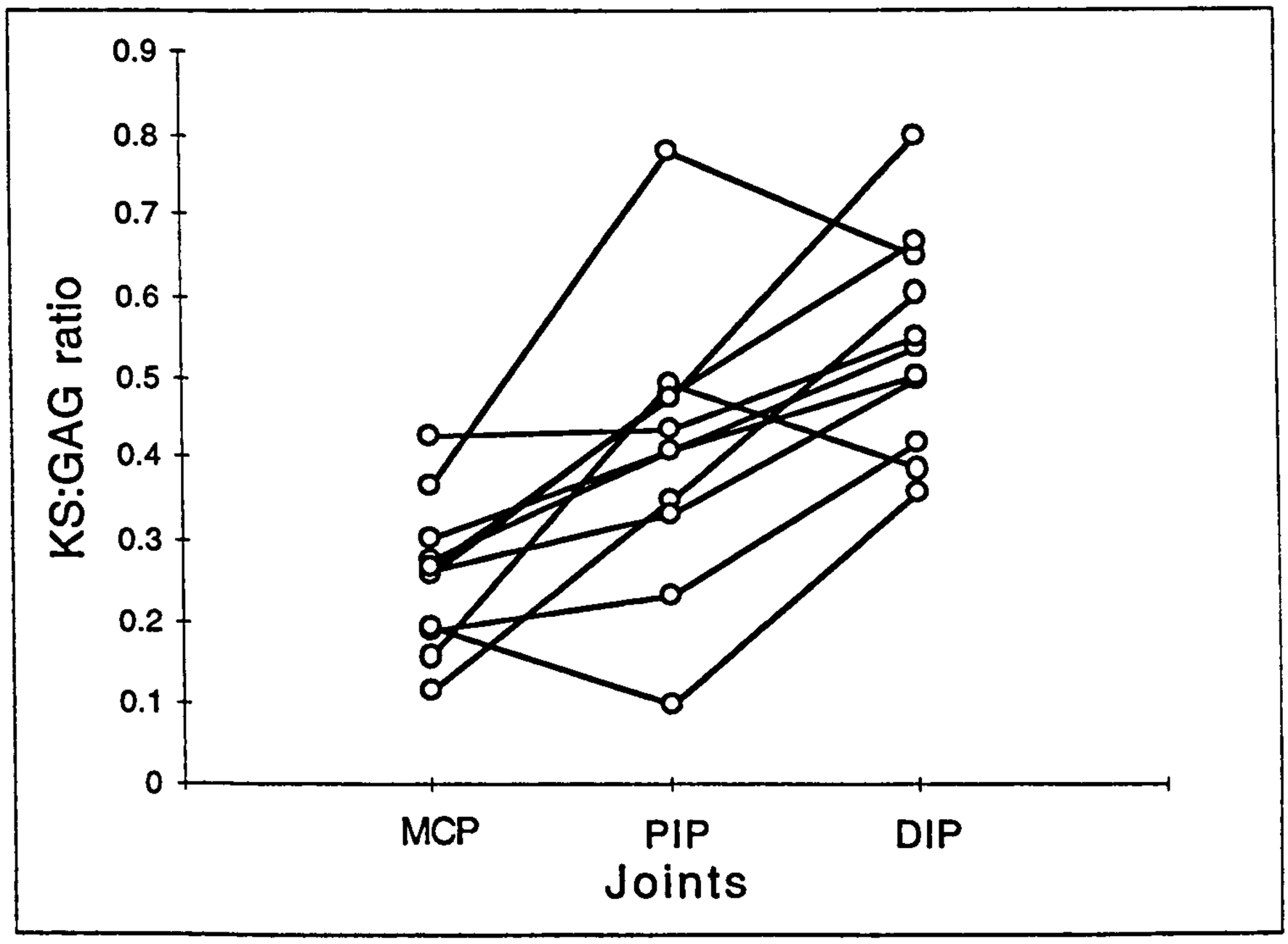

Figure 7-5a: Variation in KS : GAG ratio between normal matched joints

For abbreviations see Figure 7-3

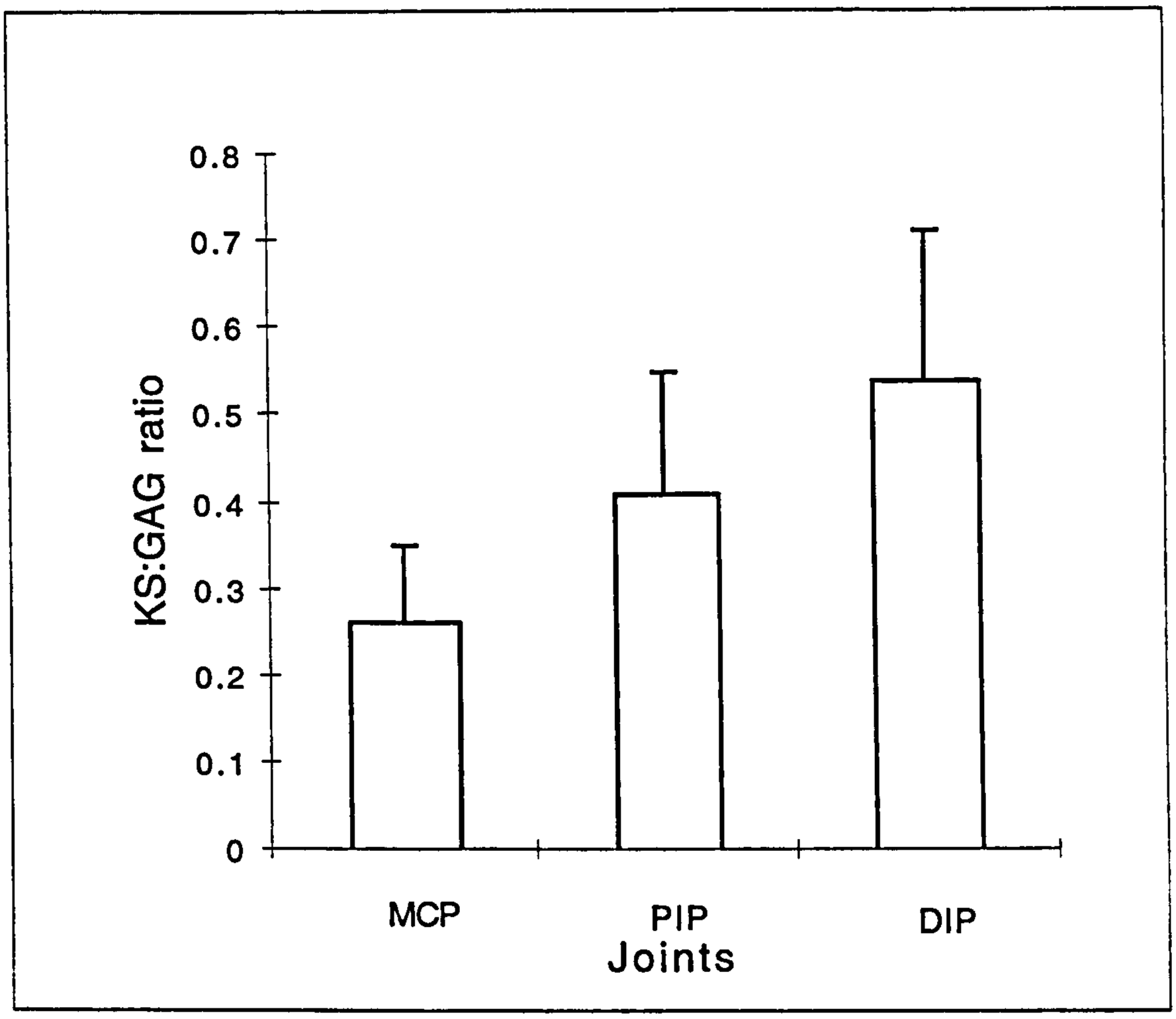

Figure 7-5b: Median KS:GAG ratios plus semi - interquartile ranges in normal matched joints.

For abbreviations see Figure 7-3

	KS	GAG	Ratio
MCP - PIP P-value	0.001	0.001	0.007
MCP - DIP P-value	0.001	0.001	0.001
PIP - DIP P-value	0.003	0.001	0.01

Table 7-3: Significance values for differences between matched joints

For abbreviations see Figure 7-3

Variation between other joints sampled.

The samples from the tarsometatarsal, tarsocrural, femoropatellar, and antebrachiocarpal joints were not matched and therefore accurate statistical analysis could not be carried out. However, although there were differences in KS and GAG values for these different joints, the ratios between these 2 measurements were nearly constant (Table 7-2) and similar to that of the metacarpophalangeal joints.

Variation with age

No significant changes were found between KS or GAG levels and age in any joint (Fig 7-6).

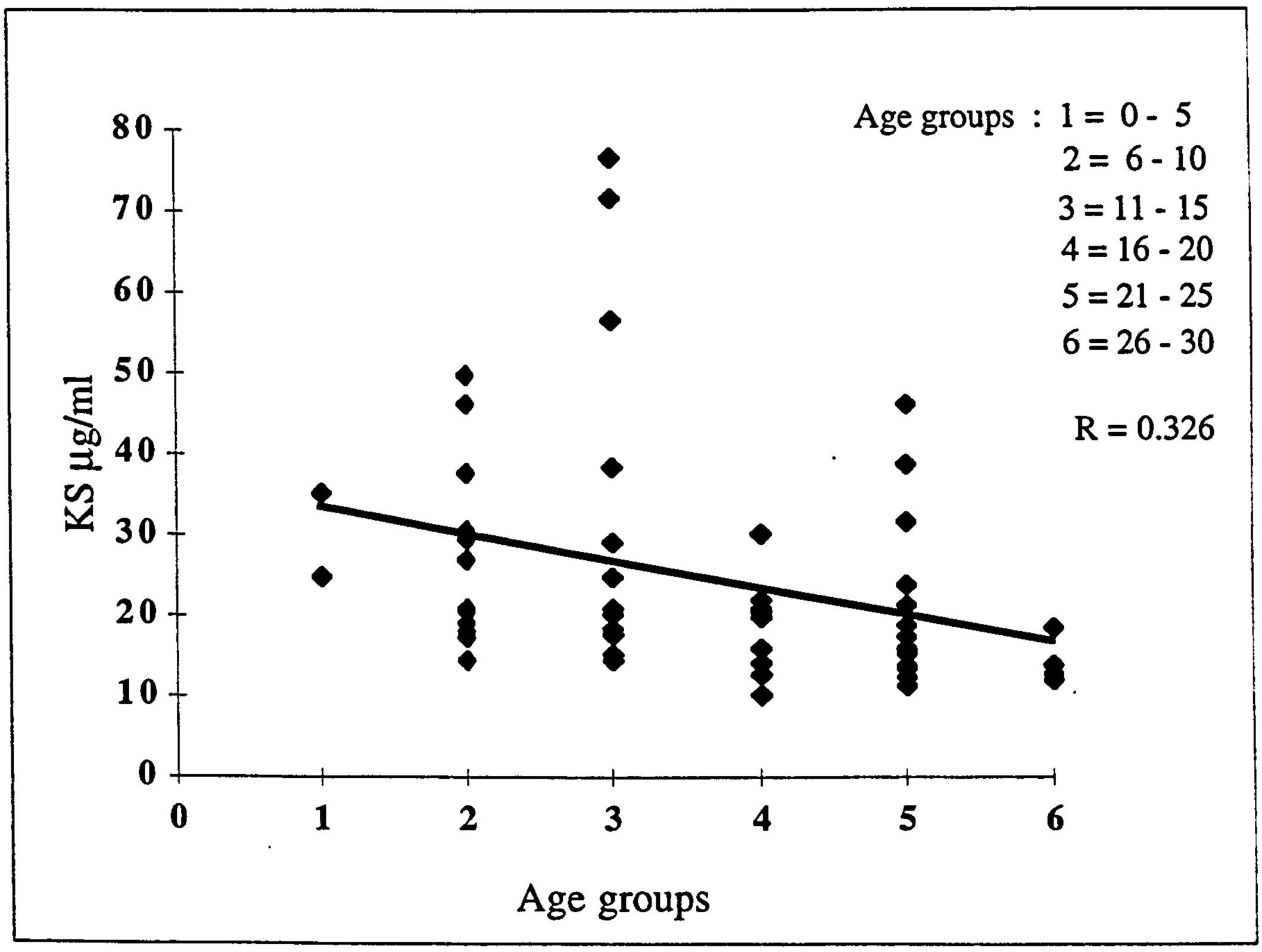

Figure 7-6: Variation of SF KS in MCP joint with age

r = -0.33 95%CI = -0.53 - 0.04 p = 0.08

Other markers measured

Marker		MCP	PIP	DIP
KS µg/ml	Median (SIQR)	19.92 (10.97)	65.62 (40.78)	144.09 (63.59)
	Range	10.24 - 76.89	15.45 - 195.80	77.59 - 272.70
GAG µg/ml	Median (SIQR)	79.21 (22.78)	165.99 (57.84)	255.62(156.92)
	Range	65.09 - 275.44	117.85- 478.78	160.45 - 505.97
COMP µg/ml	Median (SIQR)	7.61 (1.65)	11.33 (4.74)	22.55 (6.09)
	Range	4.47 - 13.88	5.29 - 27.64	13.39 - 53.29
BAP Units	Median (SIQR)	4.09 (1.15)	4.94 (1.09)	4.80 (1.18)
	Range	2.18 - 5.96	3.76 - 23.79	2.23 - 12.79
HA µg/ml	Median (SIQR)	1177 (238)	926 (247)	767 (160)
	Range	578 - 2309	612 - 1914	371 - 1785

Table 7-4: Summary of normal synovial fluid marker levels and ranges reported in this study

Abbreviations: HA = hyaluronan, COMP = cartilage oligomeric matrix protein, KS = keratan sulphate (5D4 epitope), GAG = total glycosaminoglycans,
BAP = bone specific alkaline phosphatase, SIQR = semi interquartile range

The synovial fluid samples assayed in these studies were taken from the same matched MCP, PIP, and DIP joints from the group of 11 horses used in the KS and GAG study. However because of lack of volume of some samples, the HA and the BAP studies only included samples from 10 of the 11 horses, thus altering the mean age of horses in the latter group to 17.6 years.

Cartilage oligomeric matrix protein

The median and range of COMP concentrations found in each joint are given in Table 7-4. There was a significant difference in SF concentration of COMP concentration between all joints,

i.e. MCP:PIP $p = 0.002$, MCP:DIP $p = 0.001$,
and PIP:DIP $p = 0.014$. (Figures 7-7a and b).

The differences in total COMP between the MCP and PIP and between the PIP and DIP joints were not quite significant at p=0.05 (Figure 7-7c).

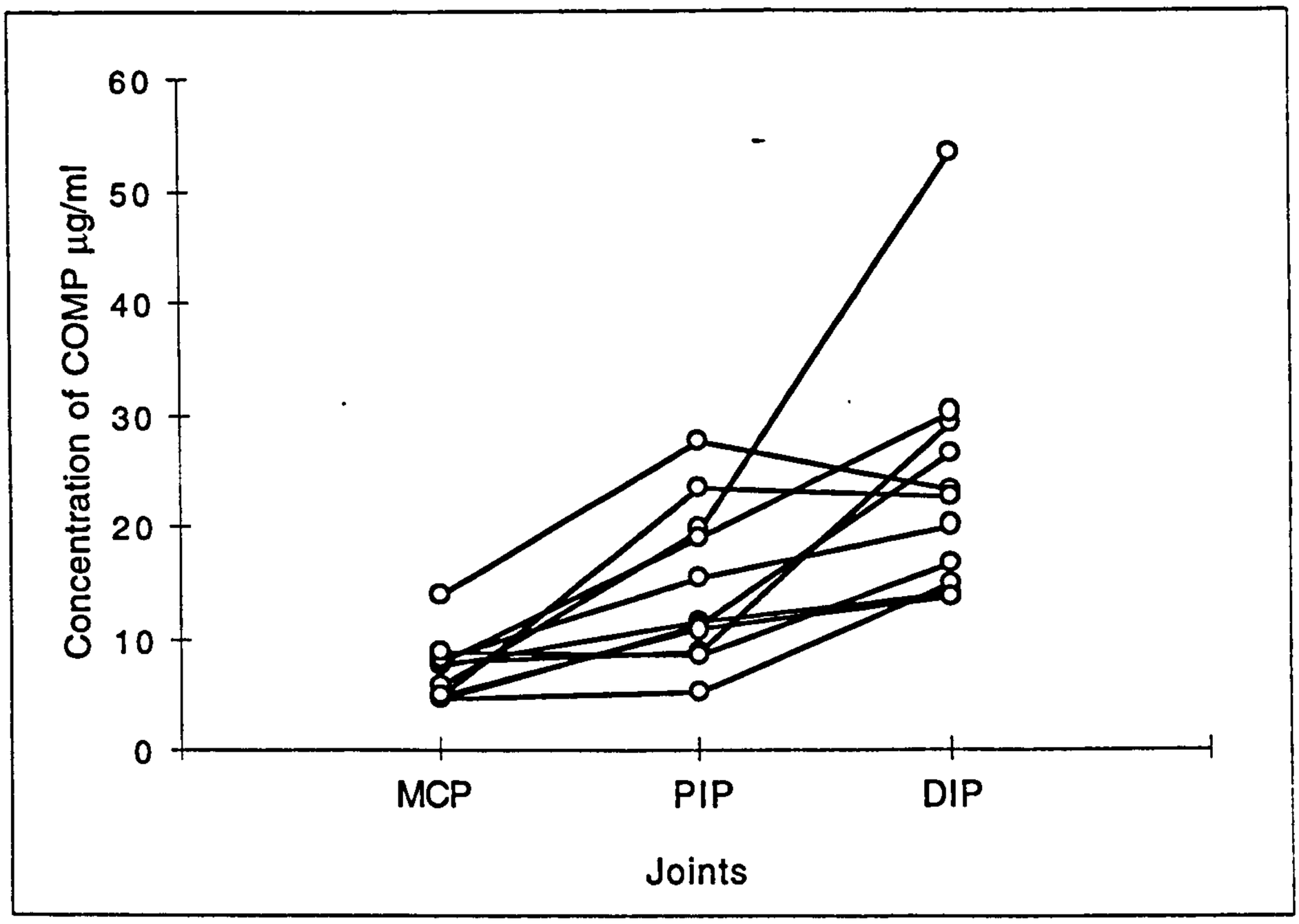

Figure 7-7a: Variation of SF COMP concentration in matched normal equine MCP, PIP, and DIP joints

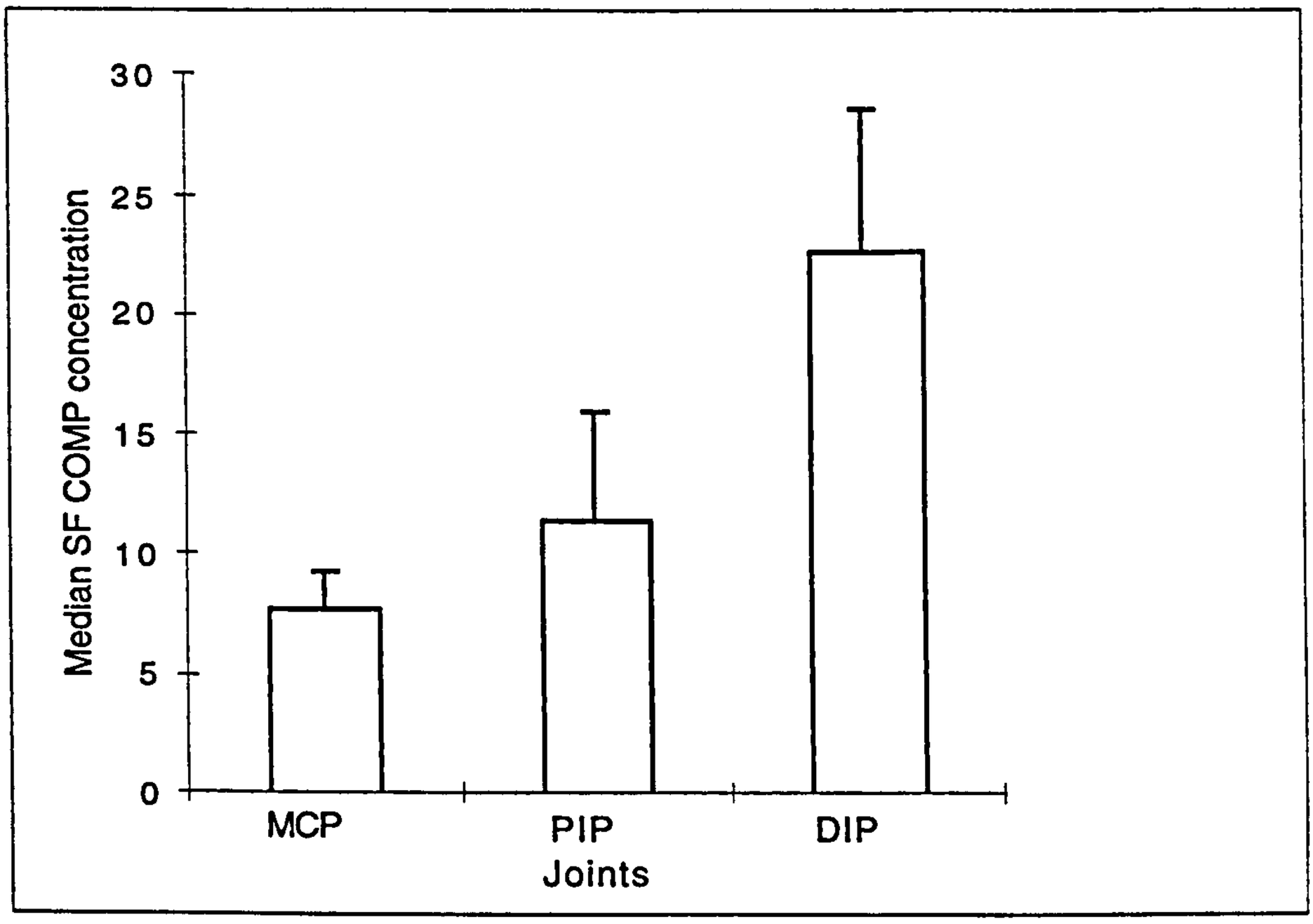

Figure 7-7b : Variation in median SF COMP concentration (plus semi interquartile range) in matched normal equine MCP, PIP, and DIP joints

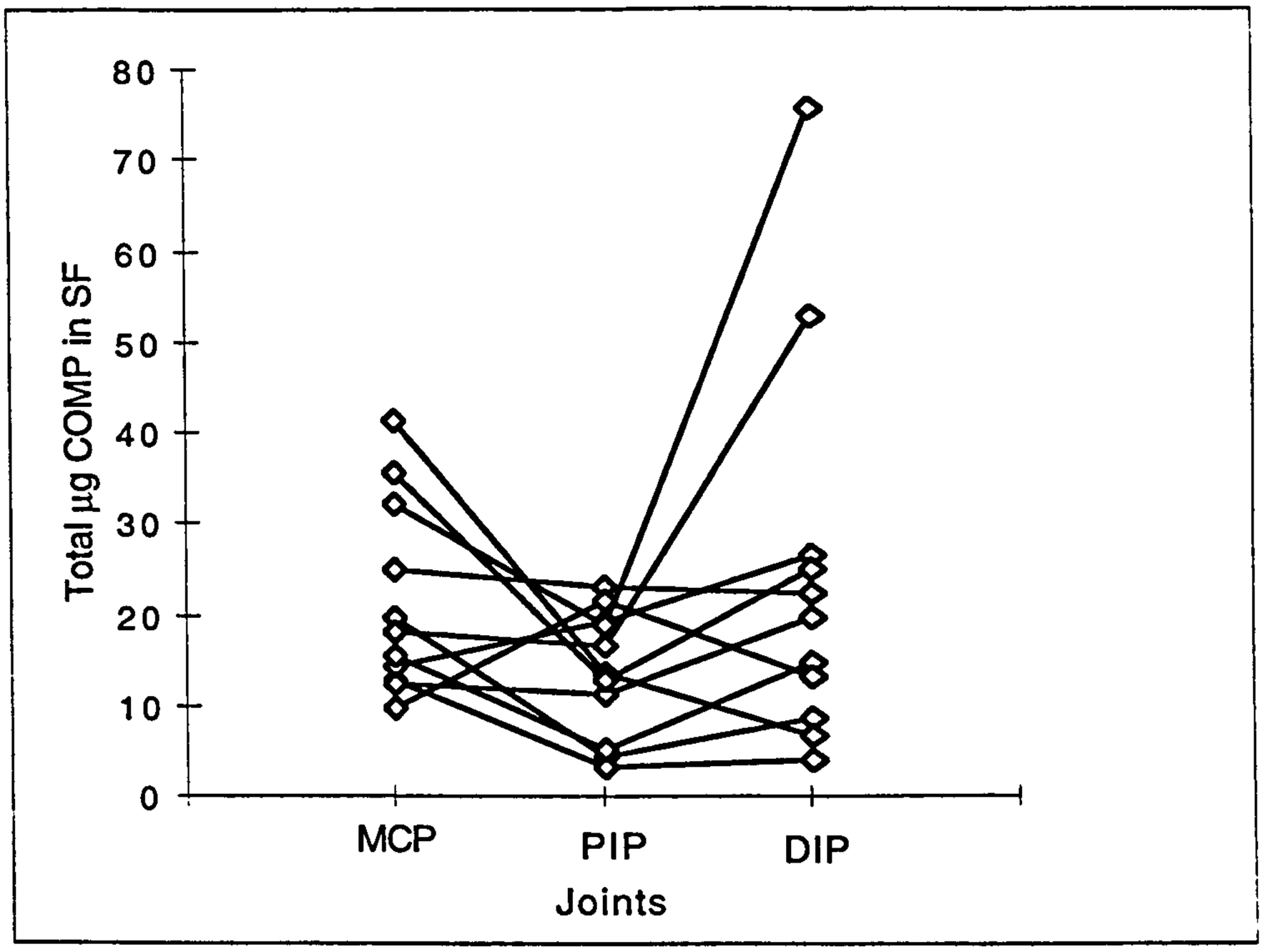

Figure 7-7c : Total µg COMP in SF from MCP, PIP, and DIP joints

There was a positive correlation between SF COMP and grouped age in the PIP joint (r_s = 0.82, 95%CI = 0.42 - 0.95, p = 0.002) (Figure 7-7d), but not in the other joints, although the COMP concentrations in the DIP joint did show a positive but not significant correlation (r_s = 0.53, 95%CI = -0.12 - 0.86, p = 0.09).

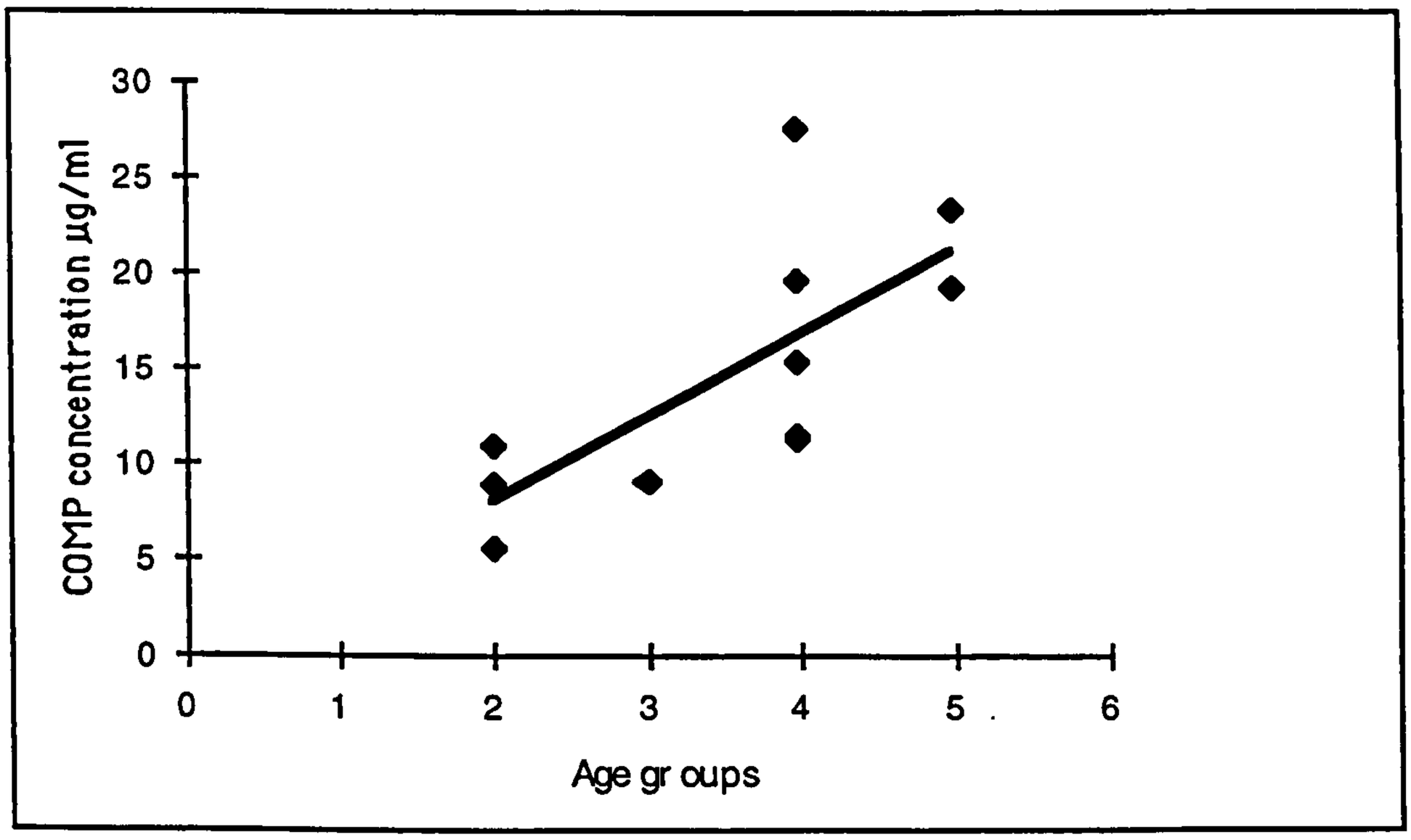

Figure 7-7d : Correlation between SF COMP in PIP joints and grouped age.

Bone specific alkaline phosphatase

The median and range of values of SF BAP are shown in Table 7-4. The difference between SF concentrations in the MCP and PIP joint only just reached significance (p=0.048) as did the difference between the MCP and DIP joint (p=0.037).

See Figures 7-8a and b.

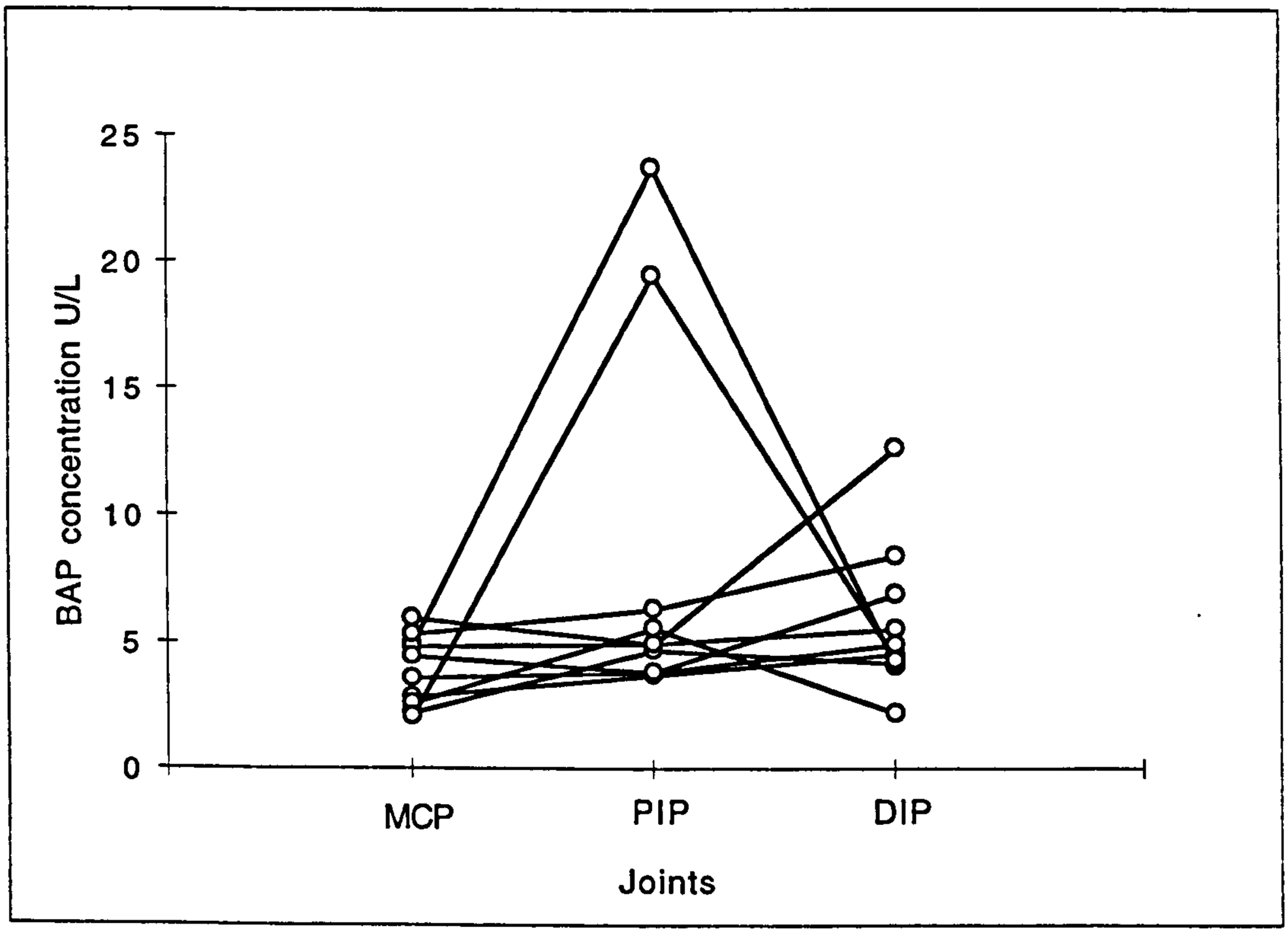

Figure 7-8a: Variation of SF BAP between matched normal equine MCP, PIP, and DIP joints.

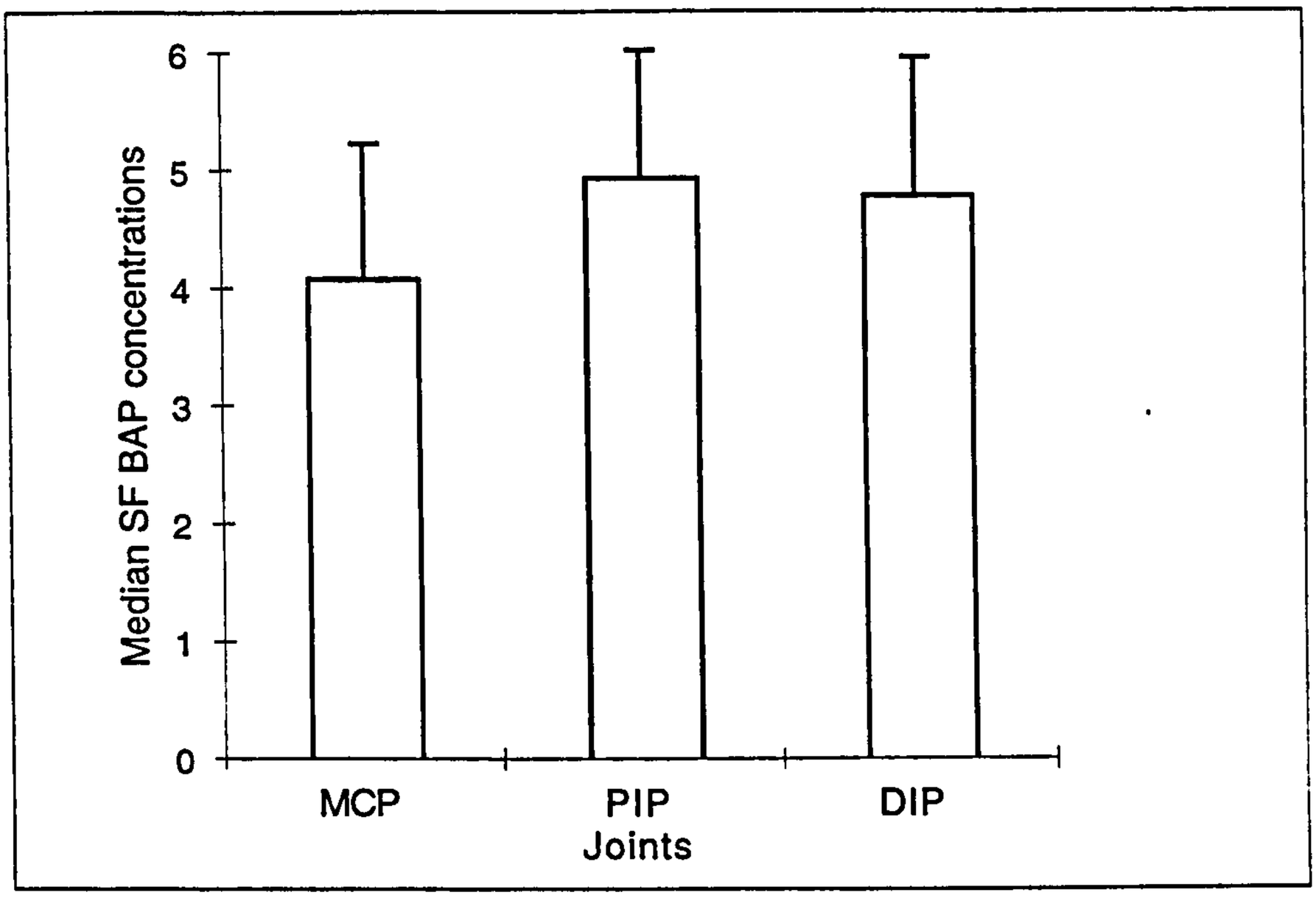

Figure 7-8b: Median (+ semi interquartile range) SF BAP concentration in matched MCP, PIP, and DIP joints.

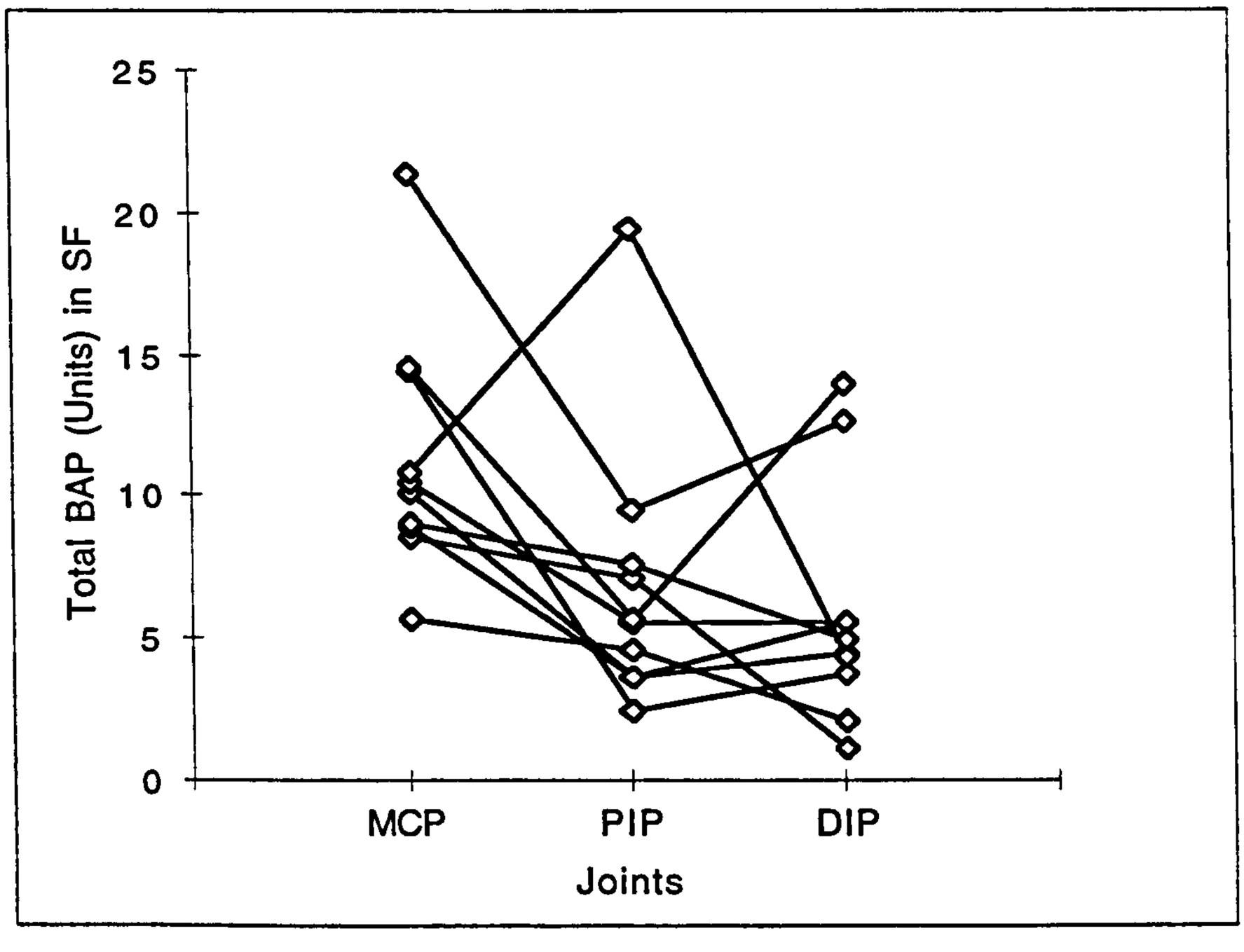

Figure 7-8c: Total BAP (units) in MCP, PIP, and DIP joints

There was a significant difference (p=0.002) (Figure 7-8c) between the total BAP in the MCP and that in the DIP joint.

There was no correlation between SF BAP and age in SF from any joint.

Hyaluronan

Synovial fluid

A significant difference was found between the synovial fluid from the MCP and PIP joints (p=0.02), and between the MCP and DIP joints (p=0.006). There was no significant difference between the concentration of HA in the PIP and DIP joints although in 8 out of the 10 pairs of joints sampled the HA concentration in the DIP joint was lower than that in the PIP joint. See Figures 7-9a and b.

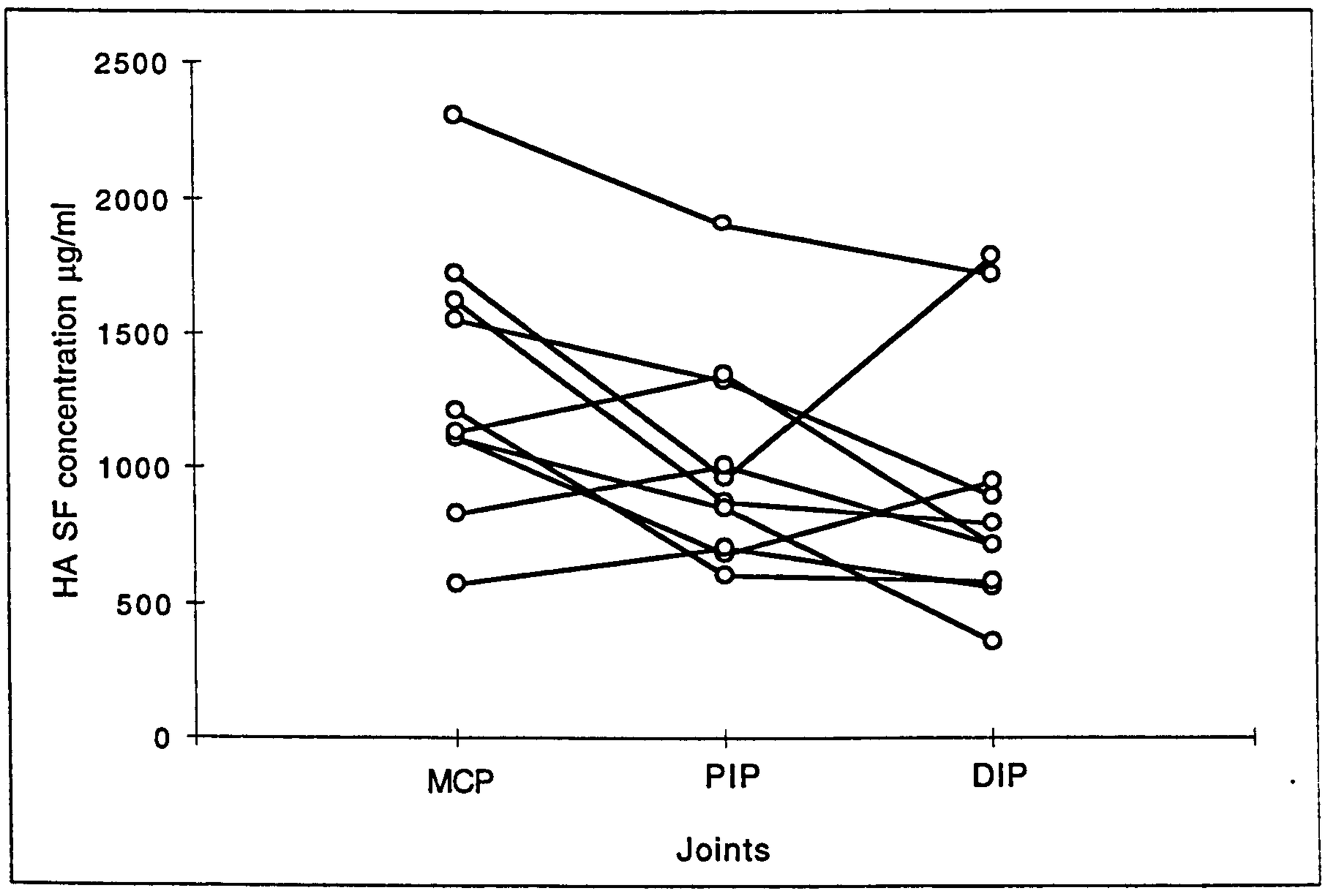

Figure 7-9a: Concentration of HASF in normal matched equine MCP, PIP, and DIP joints.

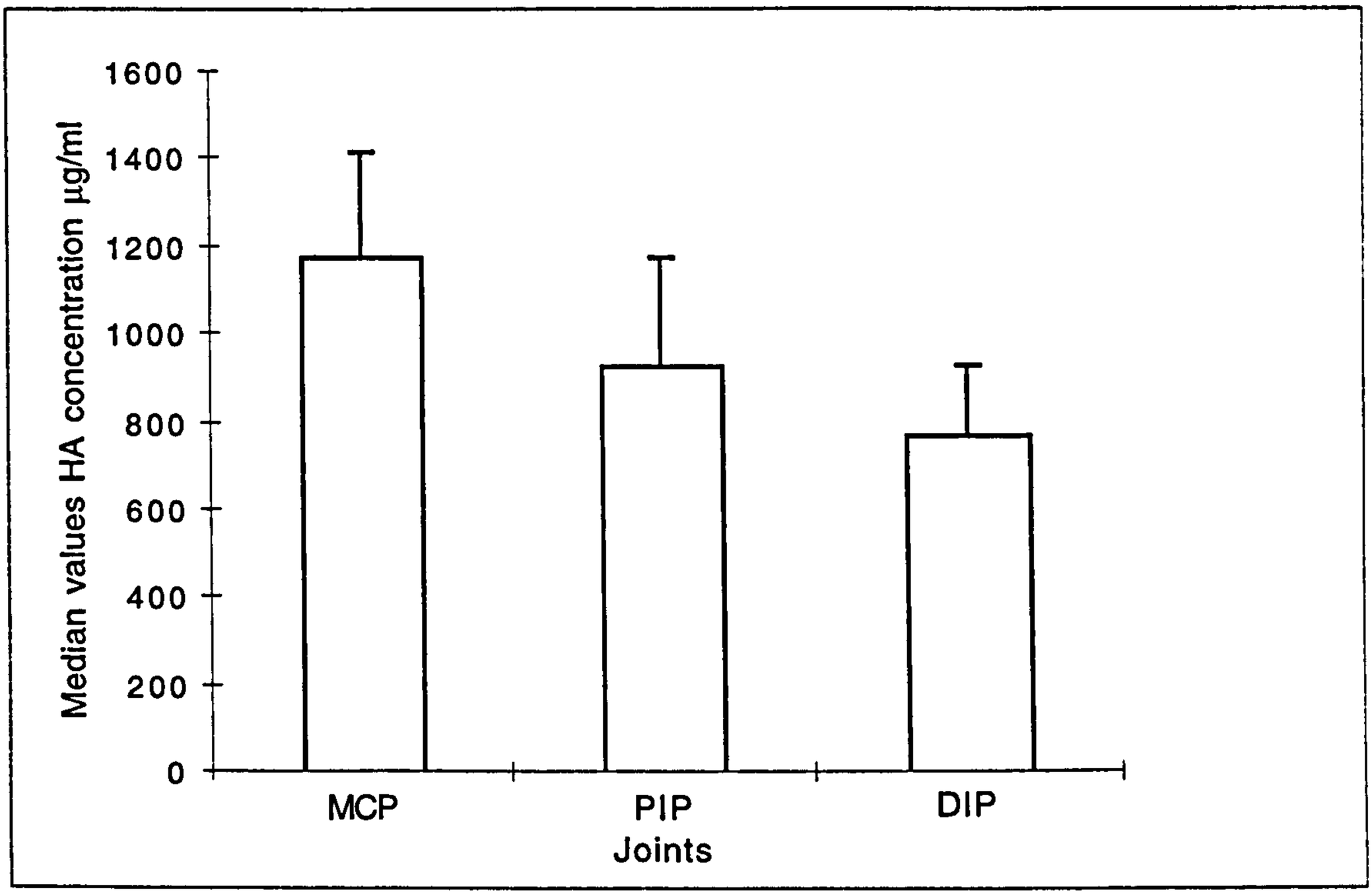

Figure 7-9b: Variation between median values (plus semi-interquartile range) of HASF in normal matched joints.

There was a positive but not significant correlation between HASF and grouped age in the MCP joint ($r = 0.58$, 95%CI = -0.08 - 0.89, $p = 0.08$) but no correlation between HA concentration and age in the other joints.

Serum - Diurnal rhythm

Horse	Breed	Age	Sex	Weight
1	Arab	15	F	450kg
2	TB	20	F	500kg
3	TB	10	F	450kg
4	Polish pony	6	F	450kg
5	TBx	4	Mn	450kg
6	Belgian Warmblood	6	Mn	550kg

Table 7-5: Age distribution and breeds of horses used in HA diurnal rhythm study.

Horse	1	2	3	4	5	6
Median µg/ml	0.23	4.69	1.51	2.17	1.04	0.37
SIQR	0.04	0.19	0.09	0.14	0.08	0.02
Range	0.19-0.37	4.27-5.63	1.22-1.82	1.93-2.59	0.88-1.46	0.33-0.46

Table 7-6: Median values and range of serum HA concentration in different horses

There was no correlation between median serum HA concentration and age in this group of horses.

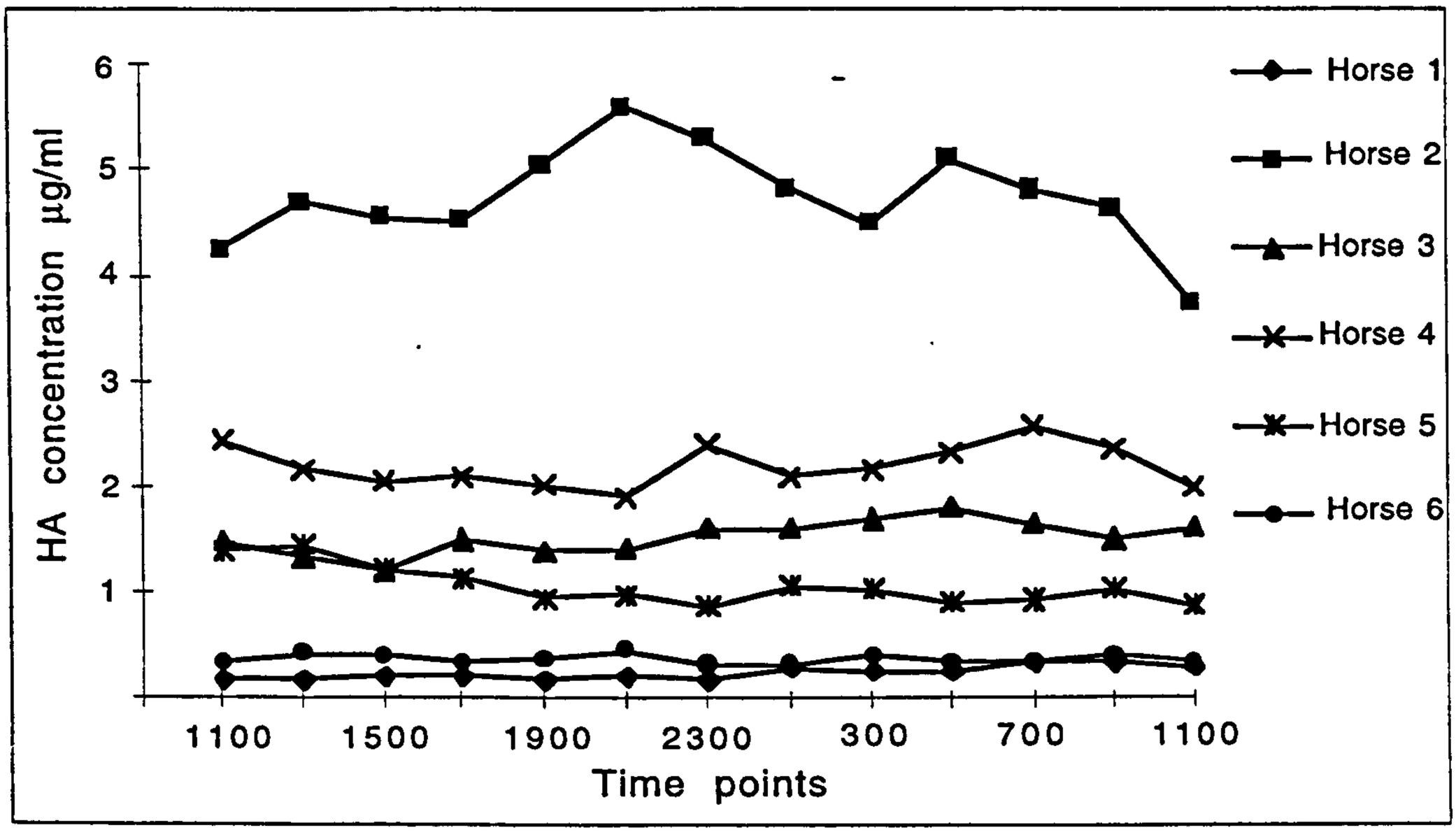

Figure 7-10a: Diurnal variation of HA concentration in plasma of six horses.

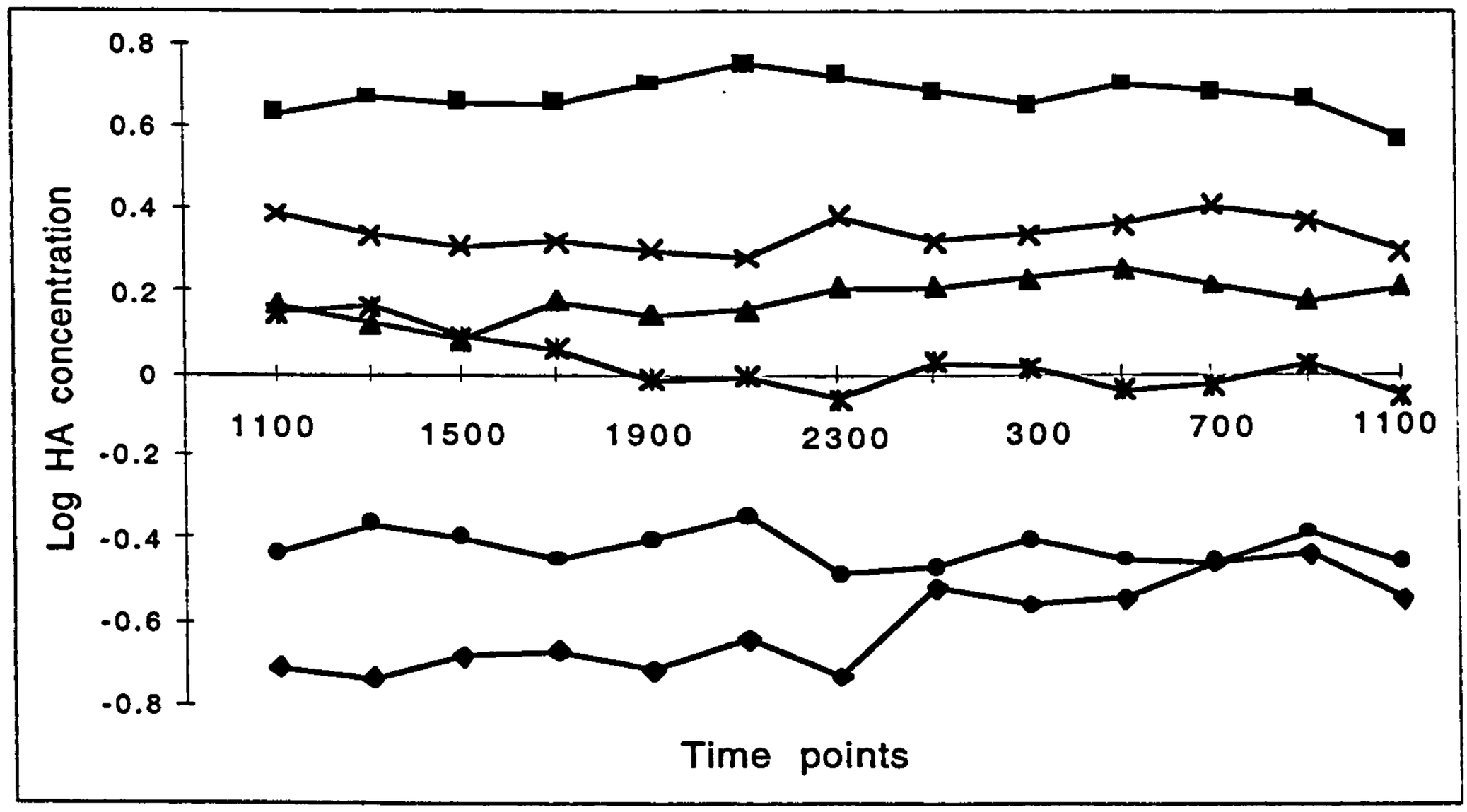

Figure 7-10b: Logarithmic transformation of diurnal plasma HA concentrations to reduce variability.

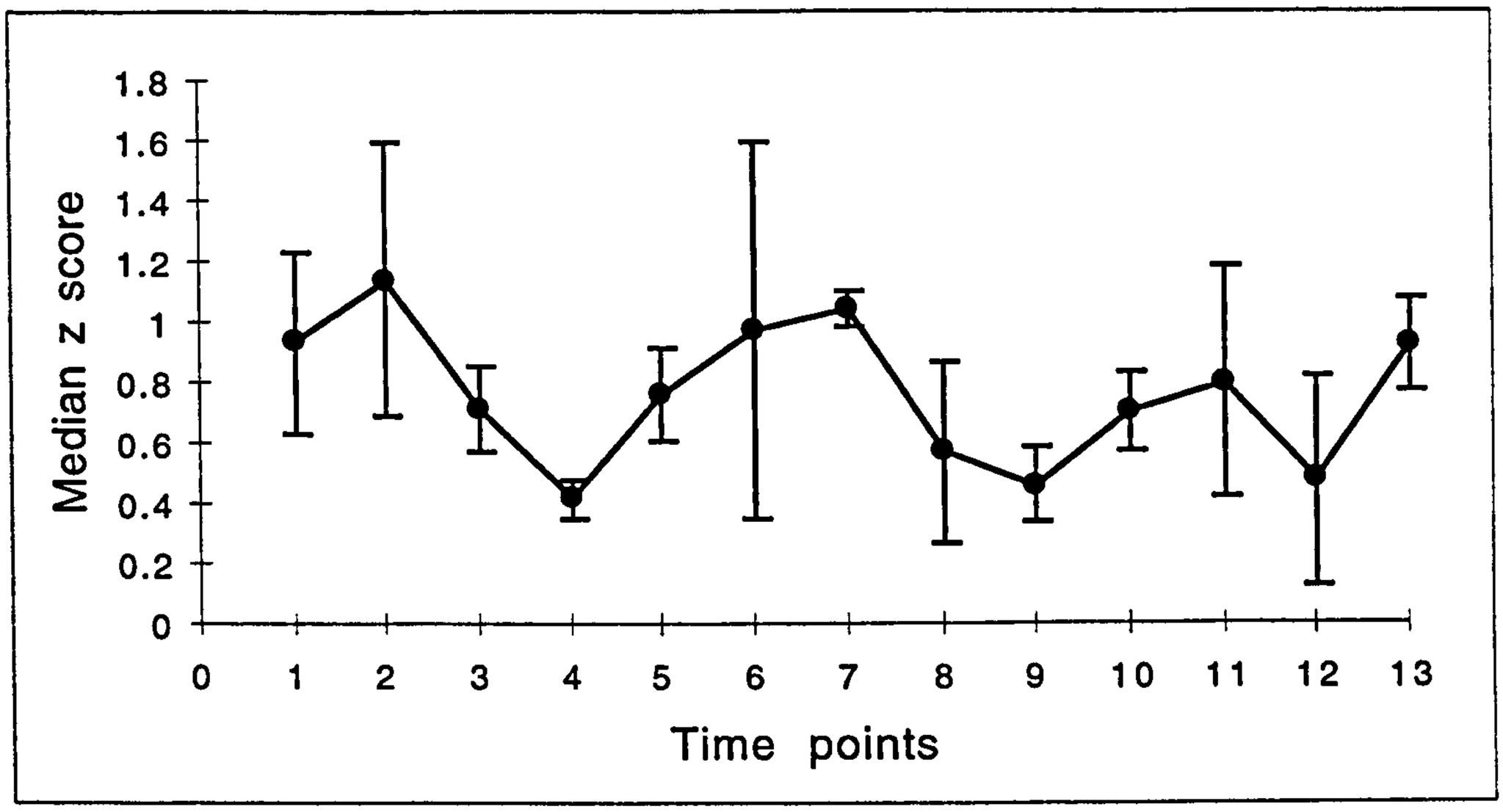

Figure 7-11a: Variation in median z scores for all six horses, plus semi interquartile range at each time point

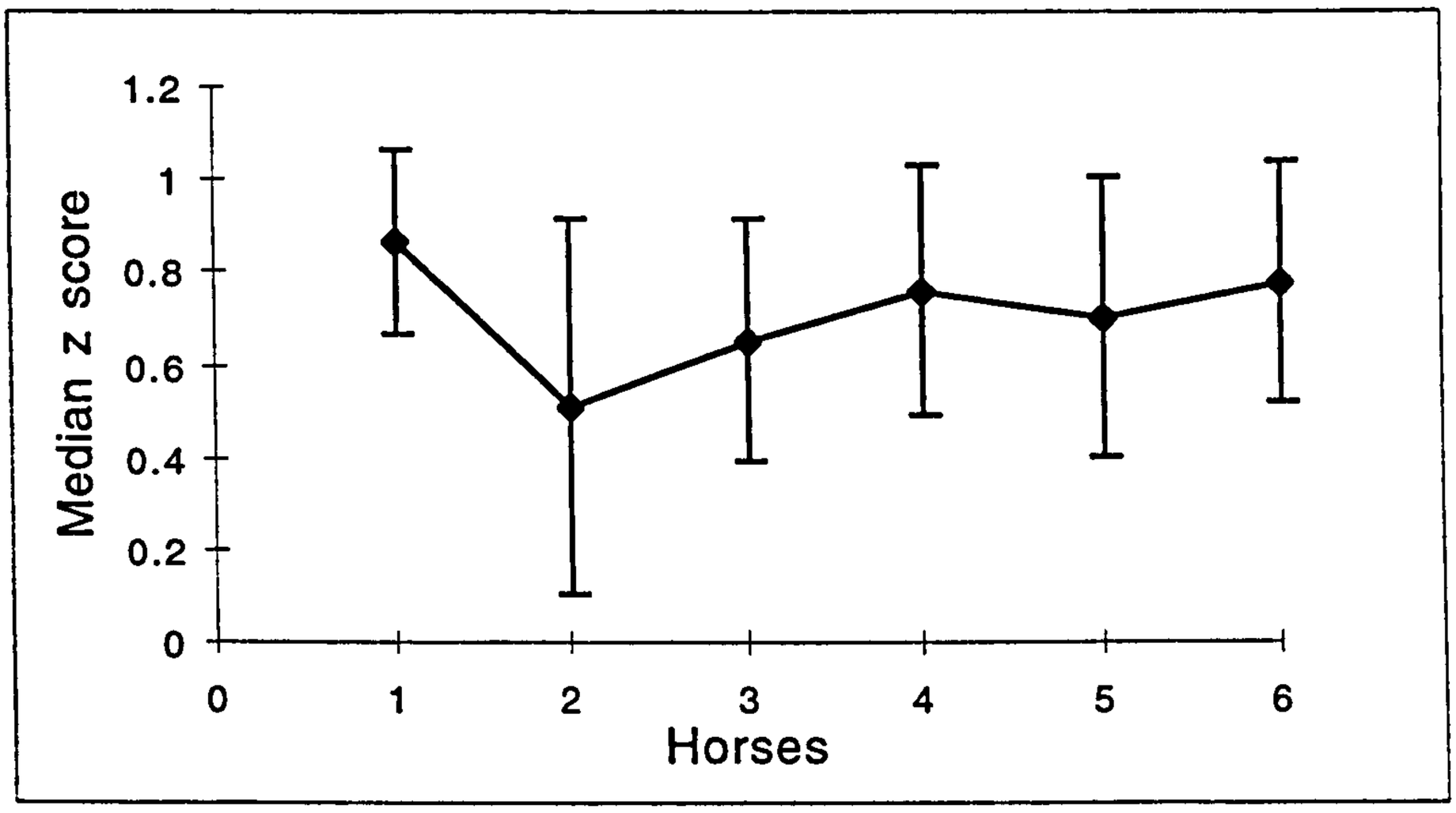

Figure 7-11b: Variation in median z scores plus semi interquartile range throughout day for each horse

Although there was a large range in the normal concentration of serum HA between horses, (0.25µg/ml - 4.75µg/ml) statistical analysis of the z-scores (value of fluctuation from the mean) for each horse and each time point showed no significant differences. The Friedman repeated measures statistic comparing Z scores at different time points was not significant , p=0.83, indicating that there was no significant fluctuation in HA concentration between different time points (Figure 7-11a) . Comparison of the fluctuation throughout the day between different horses was also not significant, the Friedman statistic being not significant at p=0.36 (Figure 7-11b). The mean coefficient of variation of all six horses was 13.7%. Therefore there appears to be no diurnal rhythm in the concentration of serum HA in the horse.

Discussion

Keratan sulphate and total glycosaminoglycans

In this study the monoclonal antibody 5D4 was used as an indicator of keratan sulphate in the synovial fluid. The highly sulphated epitope (also known as 5D4), which occurs in KS chains of greatest length and highest charge density, is antigenic to 5D4 (Caterson *et al.* 1989). The assay used therefore provides not a value for the number or mass of KS chains, but a quantity of these oversulphated sequences. This quantity is influenced by the structural presentation of the antigen, the chain length, and the degree of sulphation (Caterson *et al.* 1989; Mehmet *et al.* 1986; Poole *et al.* 1987; Seibel 1989) and these factors vary in KS from different tissues and different individuals (Hardingham *et al.* 1990; Hascall *et al.* 1987; Stuhlsatz *et al.* 1989).

In human cartilage KS chains are found to increase in number and length with increasing age up to maturity (Bayliss 1991; Stockwell 1989). No such differences have been found in bovine cartilage and no age related changes were found in this study. The majority of horses in this study (80%) were adult i.e. between 8 and 30 years old, and therefore changes occurring in the earlier years remains a possibility.

The data reported in this study show that there are differences in the KS and GAG levels between all the joints sampled. There are significant differences in the KS:GAG ratio between the distal limb joints i.e. metacarpophalangeal/metatarsophalangeal, proximal interphalangeal, and distal interphalangeal joints, but not between the metacarpophalangeal/metatarsophalangeal and the other joints sampled. These results have important implications in 2 main areas. The first is in the development of this epitope as a

marker of cartilage turnover and its potential use in the assessment of osteoarthritis. In human osteoarthritis (Sweet *et al.* 1988; Thonar *et al.* 1985; Thonar *et al.* 1992) and in equine studies (Alwan *et al.* 1990), the measurement of serum levels of KS(5D4) has been found to be useful in the assessment of cartilage turnover. However, in view of the differences found in equine synovial fluid levels of KS (5D4) in different joints, the use of KS(5D4) as a serum marker of osteoarthritis in the horse needs to be re-evaluated. If the change in synovial fluid concentration of KS (5D4) is used as the only indicator of altered joint condition, the difference in normal levels between different joints in the horse and the higher levels in the distal limb joints must be appreciated. The ratio between KS (5D4) and total GAG may be a better parameter to use, since this remains at a more constant level in all joints measured except the proximal interphalangeal and distal interphalangeal joint.

The second major implication is the significance of the variation in the levels of epitope in synovial fluid from different joints of the horse with regard to the metabolism of articular cartilage that may be occurring within these joints. There are many reasons that could account for these differences but their relative importance is unclear. They may be caused by 1) differing amounts of KS in the cartilage, or 2) differing proportions of 5D4 on the KS chains. The concentration of KS or total GAG in a joint could be affected by factors such as synovial fluid volume or cartilage mass, but these parameters in themselves are unlikely to affect the ratio of 5D4 epitope to total GAG, which is clearly seen to alter between the three distal limb joints. No evidence for selective degradation or selective clearance of KS from joints could be found in the literature.

The differences in synovial fluid analysis, reported here, provide strong evidence for an alteration in the metabolism between these joints, resulting in an increased level of KS, or expression of 5D4 epitope, in the cartilage of the proximal interphalangeal and distal interphalangeal joints. Evidence for environmental changes i.e. pH, oxygen, loading and movement of the joint having an effect on the selective expression of certain GAGs has been described (Bayliss 1991; Stockwell 1989) . For example, cartilage thickness has an effect on KS concentration, with higher levels being found in the deep regions of thicker cartilage, and those areas with decreased hydration and oxygenation. Higher proportions of KS are also found in weight bearing areas of cartilage. It could be, therefore, that environmental factors are relevant to the observed differences in KS : GAG ratio.

It is clear from the data presented that considerably more investigation is necessary in order to reach satisfactory explanations for these findings, especially with respect to the particular conditions existing within these joints.

Other markers measured

Cartilage oligomeric matrix protein

The changes reported here could be a factor of synovial fluid volume, which is largest in the MCP joint but decreases in the PIP and DIP joint. When compared to KS and GAG concentrations in the same joints (data not shown), there are no significant changes in the COMP: GAG or COMP: KS ratios between joints. The differences in COMP concentration are more likely to be correlated with mass of cartilage (See Chapter 10) - when the concentration is multiplied by the volume to provide the total amount of COMP present in the SF, the distribution is similar to that of cartilage mass differences in these same joints.

Bone specific alkaline phosphatase

There were no significant differences found in the SF from these joints. When the total units of BAP in the different joints was calculated however, there was a significant difference ($p=0.002$) between the MCP and DIP joint. This distribution cannot be connected to synovial fluid volume or cartilage mass, understandably, and the reason for this difference is not understood.

Hyaluronan

Synovial fluid

The trend in reduction of HA concentration from the MCP to the PIP and then to the DIP joint is in opposition to the changes found in the levels of KS:GAG and COMP. These changes cannot be related to synovial fluid alone since the volume of synovial fluid possible to remove on arthrocentesis was greatest in the MCP joint and decreased in the PIP and DIP joints. Synovial fluid removed from the MCP joints was always more viscous than that from the PIP and DIP joints so this result could have been expected. Viscosity is a function of both the concentration and the molecular weight of the hyaluronan. The concentration of any marker within a joint is related to the rate of synthesis, degradation, and mobilisation of the marker from that joint. There is no evidence for differences between the rates of synthesis and degradation in different joints, but since the rate of clearance will be affected by molecular size, it is possible that larger molecular weight chains are cleared to the lymphatics more slowly and therefore result in an increased concentration of the molecule within the joint. It has been shown that the turnover of HA in the carpal joints is more rapid than that in the MCP joints and this finding has been attributed to workload of the joint, range of motion, vascularisation and anatomy of the synovial membrane (Lindholm *et al.* 1996). There is also evidence for more rapid metabolism in smaller joints (Ekman *et al.* 1981). It seems likely therefore that

on or a combination of these factors is the reason for the synovial fluid differences detected here.

Correlation between COMP, BAP, and HA with age in the SF.

There was a significant correlation between COMP and age in the PIP joint only. There was no correlation between SF BAP and age in any joint. Apart from a trend towards significant correlation between HA and age in the MCP joint, no other significant correlations were found. It is known that the concentration of HA in cartilage increases with age (Holmes *et al.* 1988) but release of HA from the cartilage is only of minor importance to the SF content (Hedin *et al.* 1991) so it is unlikely that this would affect any possible correlation. It seems that in this study there were too few horses with too small an age range to make any firm conclusions.

It appears that although the reasons for the differences in marker concentrations between these joints remains unclear, the fact remains that differences do exist, and these must be taken into account when interpreting marker concentrations. The range of normal values for all the markers identified here will serve as an essential baseline from which to compare changes in the OA joint.

HA Diurnal rhythm

Unlike previous findings in man and rat there appeared to be no evidence of diurnal rhythm in the horse. In man the increased levels of serum HA occuring following morning rising are believed to be due to increased mobilisation of HA from the joints after a period of rest. The behaviour of horses, sleeping only for short periods and often in the standing position may preclude this effect. It was possible that the disturbance of the horses in order to take the blood samples on each occasion may have affected the results, but apart from keeping the animals stabled there was no other way to reduce movement practically. The large range of values found in each horse during a 24 hour period, plus the large range of values between normal horses, implies the need for caution when interpreting serum HA levels. One horse (2) had a much higher serum level. This was the eldest horse in the group, and serum HA is known to relate to age (Tulamo *et al.* 1990). With age, more HA of lower molecular weight (MW) is synthesised, and since the liver has an affinity for higher MW molecules, these lower MW HA remain in the circulation longer thus increasing the concentration (Tengblad *et al.* 1986). It is also known that even light walking can affect the serum HA (Tulamo *et al.* 1990), however there was no correlation in this study between the HA levels and the more active horses.

Summary

1. A normal range of values for KS, GAG, HA, COMP and BAP in equine synovial fluid and for HA in serum has been established. Differences in the normal concentration of KS, GAG, and more importantly the KS:GAG ratio, HA, and COMP have been demonstrated in different equine joints.

2. The variation of these markers with age remains inconclusive.

3. A normal range of HA in equine serum has been established, and found vary widely between horses.

4. There does not appear to be a diurnal rhythm of serum HA in the horse.

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Chapter Eight

Biochemical markers in osteoarthritis :

Cross-sectional studies

Introduction

The background to the markers measured here is detailed in Chapter 7. Having investigated the ranges and variations of these biochemical markers in normal joints, in this chapter the changes in these markers which occur in osteoarthritis (OA) are explored.

1. Serum markers

In human studies the level of serum hyaluronan (HAS) has been found to correlate with the radiographic OA score in the knee (Campion *et al.* 1991) and with clinical joint symptoms (Takei *et al.* 1996). In OA in man HAS has been found in increased levels (Campion *et al.* 1991), and it has also been shown to be predictive of OA progression (Sharif *et al.* 1995). However, results from an equine study indicated that there is a large range of normal values for HAS between horses and serum levels can be affected by even mild exercise (Tulamo *et al.* 1990). In both horse and man HAS correlates positively with age (Campion *et al.* 1991; Tulamo *et al.* 1990). No diurnal rhythm has been found in the horse (Chapter 7).

2. Synovial fluid markers

Synovial fluid hyaluronan (HASF) has been reported to be lower in equine OA joints when compared to normal controls (Hilbert *et al.* 1984). No correlation has been detected between HASF and the degree of lameness (Tulamo *et al.* 1994) but a negative correlation has been reported between articular cartilage damage and the HASF concentration (Tulamo *et al.* 1996). The 5D4 epitope of keratan sulphate (KS (5D4)) is lower in equine OA joints (Todhunter *et al.* 1997) than in normal controls and can be affected by breed and gender of horse. Total glycosaminoglycans (GAG) has been reported to be higher in OA joints when compared to normal controls in animals (Alwan *et al.* 1991; Arican *et al.* 1994) and in man (Saxne *et al.* 1987). No correlation has been detected between GAG and articular cartilage damage (Tulamo *et al.* 1996).

In a canine study bone specific alkaline phosphatase (BAP) was found to be increased in actively OA stifle joints when compared to the contralateral joint but no correlations were found between BAP concentration and background variables (Innes 1997).

Since the degeneration of articular cartilage has long been accepted as the pivotal diagnostic feature of OA, correlation between the degree of cartilage damage and the change in concentration of any biochemical marker from normal, in serum or synovial fluid (SF) will obviously give criterion validity to that marker. Arthroscopy, although more invasive than radiography or magnetic resonance imaging, allows direct visualisation of the cartilage, and various methods of scoring the degree of damage have been described. Many have not been accepted because of their complex nature, especially regarding the number of cartilage locations assessed, the determination of cartilage lesion diameter, and the often poor definition of depth criteria. A suitable system must be simple enough to be highly reproducible both intra and inter-observers., and grading decisions should be unambiguous and objective (Marshall 1996). Ayral (Ayral *et al.* 1996) found global arthroscopy scoring using a visual analogue scale to correlate well with the Societe Francaise d'Arthroscopie grading system - a composite index accounting for depth, size, and localisation of cartilage lesions, thereby suggesting good intrinsic validity of the arthroscopic variables. The arthroscopy score correlated well with radiographic variables. Intra-observer variation was good, and better than the variation inter-observer. Longitudinal change in score correlated significantly with functional changes. In 1996, Ratcliffe *et al* (Ratcliffe *et al.* 1996) reported that in an arthroscopic study of the glenohumeral joint in man, using a global grading scheme, measurement of GAG, KS, and 3B3(-) correlated well with the arthroscopic diagnosis of OA. Most scoring systems in the veterinary literature rely on the increase percentage loss of cartilage in the joint without taking account of the localisation of lesions (McIlwraith *et al.* 1987) (Tulamo *et al.* 1996). Results from the veterinary studies have been variable. In one study comparing arthroscopy and radiology in assessment of carpal OA (Kannegieter *et al.* 1990) correlation between the two measurements was poor, while in another study radiological changes in subchondral bone were found to correlate well with arthroscopic findings (Steinheimer *et al.* 1995). The degree of cartilage damage in the joint has been reported to correlate negatively with the HASF concentration (Tulamo *et al.* 1996) in the horse.

Once the criterion validity and normal range of a marker have been assessed, any variation of that marker with background variable e.g. age, duration of OA, and exercise must be established to allow meaningful interpretation of changes in marker levels in disease states. In chapter 7, the variation of normal marker levels with age was investigated but without conclusive results. In this chapter, correlation between markers and age and duration of OA will be re-examined.

Aims

1. To investigate the cross-sectional differences existing between biochemical markers in normal and OA synovial fluid and serum in two different populations of horses with early, and later stage, OA.

2. To ascertain whether any relationship exists between the markers and the level of articular cartilage damage in the joint.

3. To investigate the correlations between markers and background variables.

Methods

During this study, synovial fluid and serum samples were collected from three different groups of horses. The first group was a sample of Thoroughbred racehorses in training, which underwent arthroscopic examination of their carpal joints after short periods of lameness. The second group were horses diagnosed with OA after referral to the Department of Clinical Veterinary Science, University of Bristol. The third group was the selection of 20 horses recruited to the CaPPS clinical trial. The second and third groups, being of similar types and duration of OA, were grouped together for analysis.

1. Arthroscopy cases

Twentytwo horses, the majority of which were Thoroughbreds in racing training underwent arthroscopic examination of their carpal joints following recent onset lameness. In each case the degree of cartilage damage in each joint was scored, by the same observer in each case, according to a scale adapted from that used by McIlwraith (McIlwraith *et al.* 1987) (Table 8-1). Synovial fluid, from both the clinically affected and the contralateral joint, and serum samples were collected at the time of surgery, while the horses were under anaesthesia. These samples were processed for storage and analysis as described in Chapter 7. Normal serum controls used in this study were taken from 15 racehorses in training which showed no clinical signs of lameness.

Score	Degree of articular cartilage damage
0	Normal.
1	Minimal articular cartilage degeneration.
2	Articular cartilage degeneration affecting up to 30% of the articular surface of the bone.
3	Loss of up to 50 % of the articular cartilage from the affected carpal bone.
4	Severe loss of cartilage affecting more than 50% of the articular surface.

Table 8-1: Scoring system used for the assessment of degree of cartilage damage

Hyaluronan (HA) was measured in all serum samples, and HA, 5D4 epitope of keratan sulphate (KS (5D4)), total glycosaminoglycans (GAG) and bone specific alkaline phosphatase (BAP) was measured in all SF samples. Assay methods are described in Chapter 7.

2. OA cases

Samples of serum and SF were collected from horses for which the cause of lameness had been diagnosed as OA. Diagnosis of OA was made as described in Chapter 2 i.e. by a combination of clinical examination, intra-articular analgesia, radiography, and in some cases scintigraphy. Included in this cohort were the horses recruited for the CaPPs trial, from which the samples collected at their first examination were included in the cross-sectional marker investigation. Normal synovial fluid samples used for controls in this study were those used in the study of normal joint variation (Chapter 7). Normal serum controls were from the six horses used in the diurnal rhythm study (Chapter 7) and from four other horses resident in the Department of Clinical Science which showed no clinical signs of locomotor disease. HAS and HASF, KS(5D4), GAG, and BAP were measured in all samples.

Statistics

Distribution of markers were found to be skewed, even after logarithmic transformation, so for statistical analysis, nonparametric methods were used. In comparison of markers in paired joints in the arthroscopy group, Wilcoxon signed rank tests were used. For comparison of different groups the Mann Whitney U test was used, and correlations were made using the Spearmans correlation coefficient (r_s). Values were considered significant at or below 0.05, and confidence intervals were stated at 95%.

Results

1. Arthroscopy data

Out of 22 horses which underwent bilateral arthroscopic examinations, paired synovial fluid samples were available from 19 cases. Serum was available from 15 of these cases. The mean age of the horses in this group was 3.6 years and the duration of lameness in any case did not exceed 3 months. 17/22 horses were Thoroughbreds in racing training. Of the 22 pairs of joints examined, 15 were midcarpal (MC), 4 were antebrachiocarpal (ABC), and 3 were metacarpophalangeal (MCP) (Table 8-2).

Case	Age	Breed	Joint	Left score	Right score
1	?	?	MC	1	1
3	2	TB	MC	2	1
4	9	Arab	MCP	0	2
5	4	TB	MCP	0	2
6	?	Hunter	ABC	2	0
7	3	TB	MC	2	1
8	7	TBX	ABC	1	0
10	4	TB	MC	2	1
14	2	TB	MC	3	1
15	2	TB	MC	1	2
16	4	TB	MC	3	1
17	2	TB	ABC	2	1
18	2	TB	MC	2	1
19	2	TB	MC	2	1
20	?	TB	MC	1	3
21	?	TB	MCP	1	0
22	5	TB	MC	3	0
23	?	TB	MC	0	0
24	3	TB	MC	1	0
25	3	TB	MC	0	3
28	13	Conn	ABC	3	0
29	4	TB	MC	3	0

Table 8-2: Arthroscopy cases with scores of cartilage damage.

Abbreviations:
TB = Thoroughbred, TBX = Thoroughbred Cross, Conn = Connemara
MC = midcarpal, ABC = antebrachiocarpal, MCP = metacarpophalangeal.

Synovial fluid markers

a) Hyaluronan

Paired samples from 18 cases were analysed for HA. The median HA concentration plus the semi interquartile range (SIQR) in clinically active joints was 741.6 µg/ml (222) and in the contralateral joints was 1061.75 (325) µg/ml.

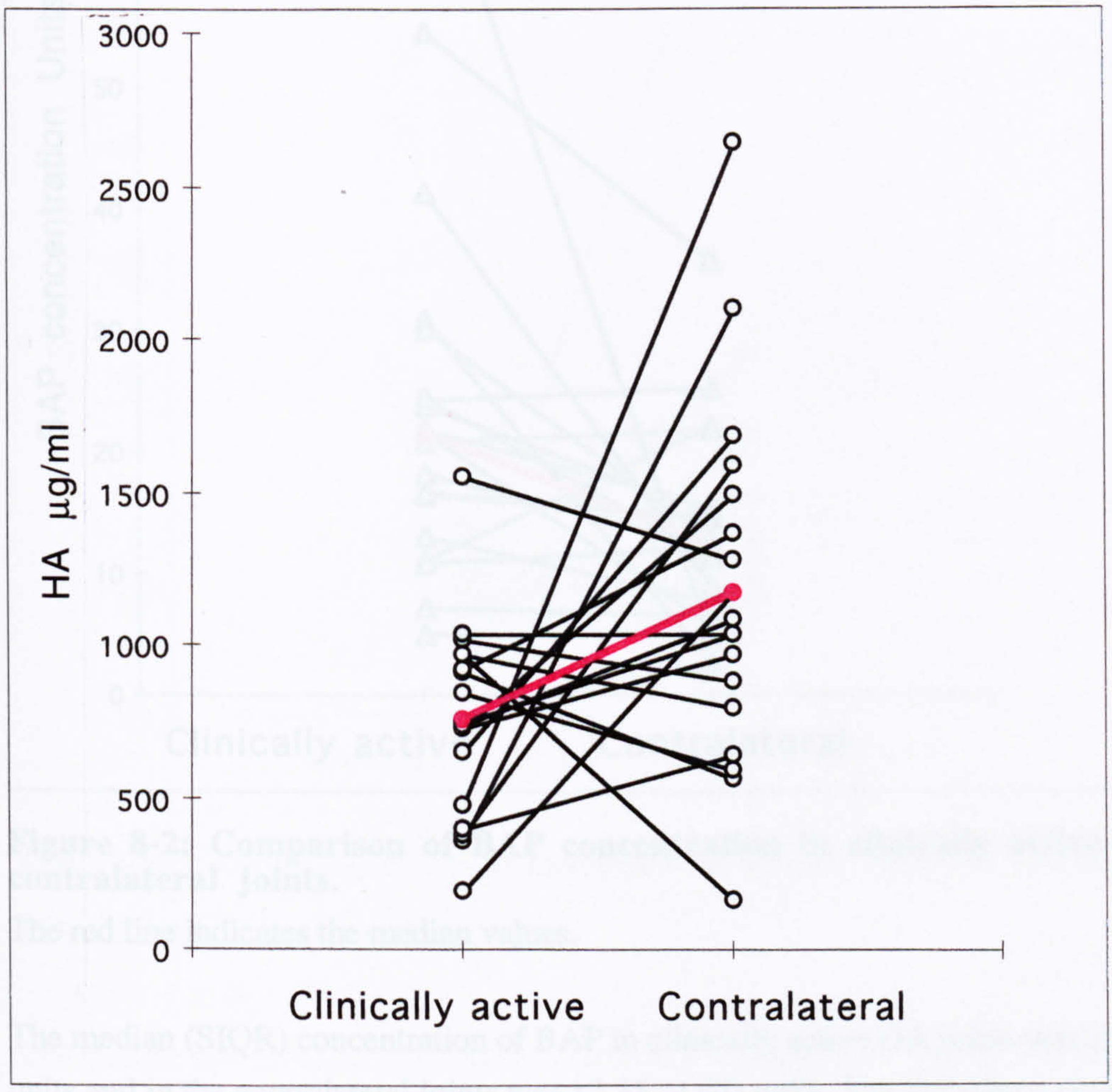

Figure 8-1: Comparison of HASF concentration between paired clinically active and contralateral joints in arthroscopy group.

The red line indicates the median values.

There was a significant increase in HASF concentration from the clinically active OA to the contralateral joint , p = 0.01, (Mann Whitney - U test)

b) Bone specific alkaline phosphatase

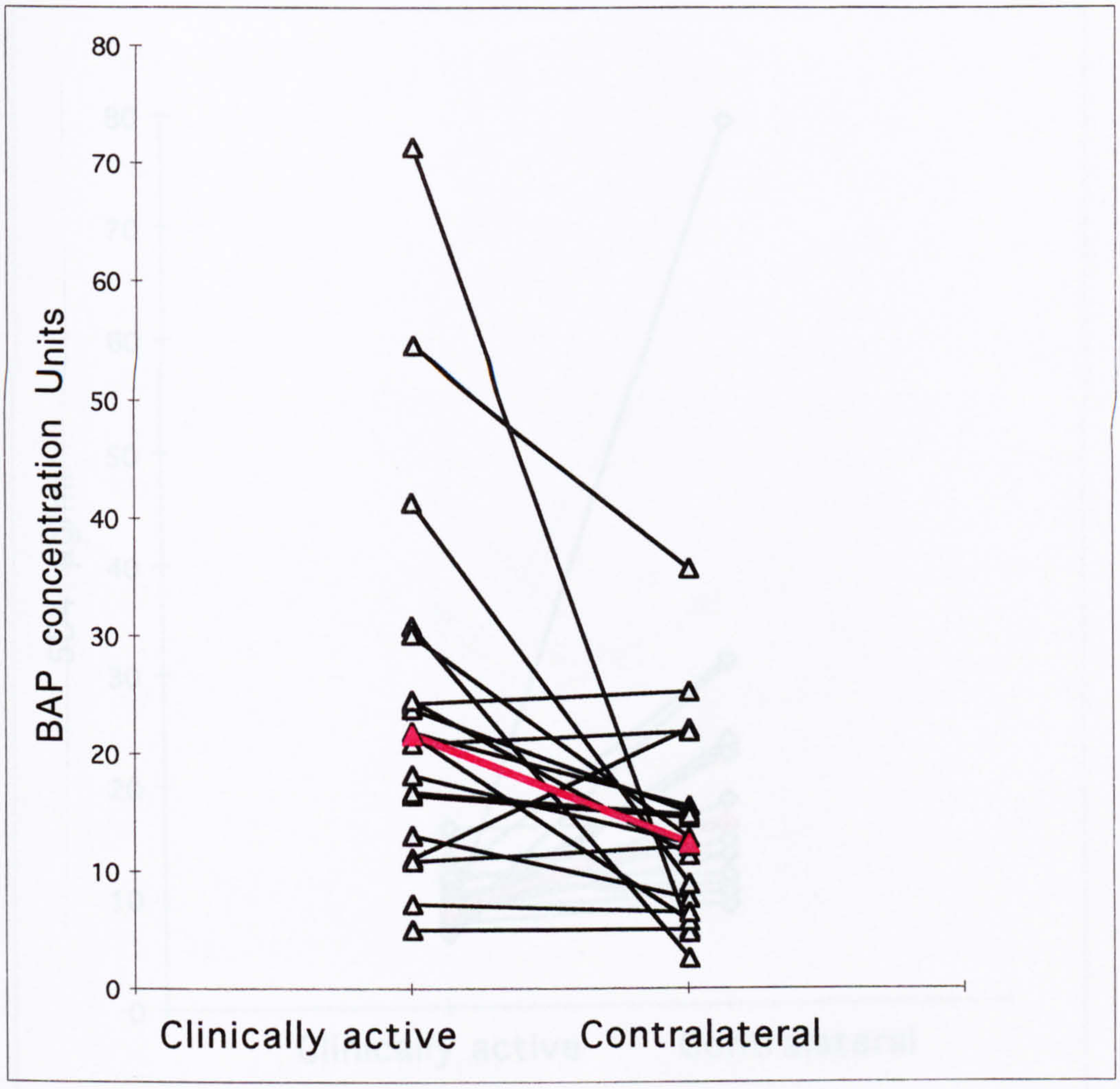

Figure 8-2: Comparison of BAP concentration in clinically active OA and contralateral joints.

The red line indicates the median values.

The median (SIQR) concentration of BAP in clinically active OA joints was 21.75 (6.22) units and in the contralateral joints was 12.35 (4.07) units. The difference between the joints was significant , p = 0.002 (Wilcoxon signed rank).

c) *Keratan Sulphate (5D4)*

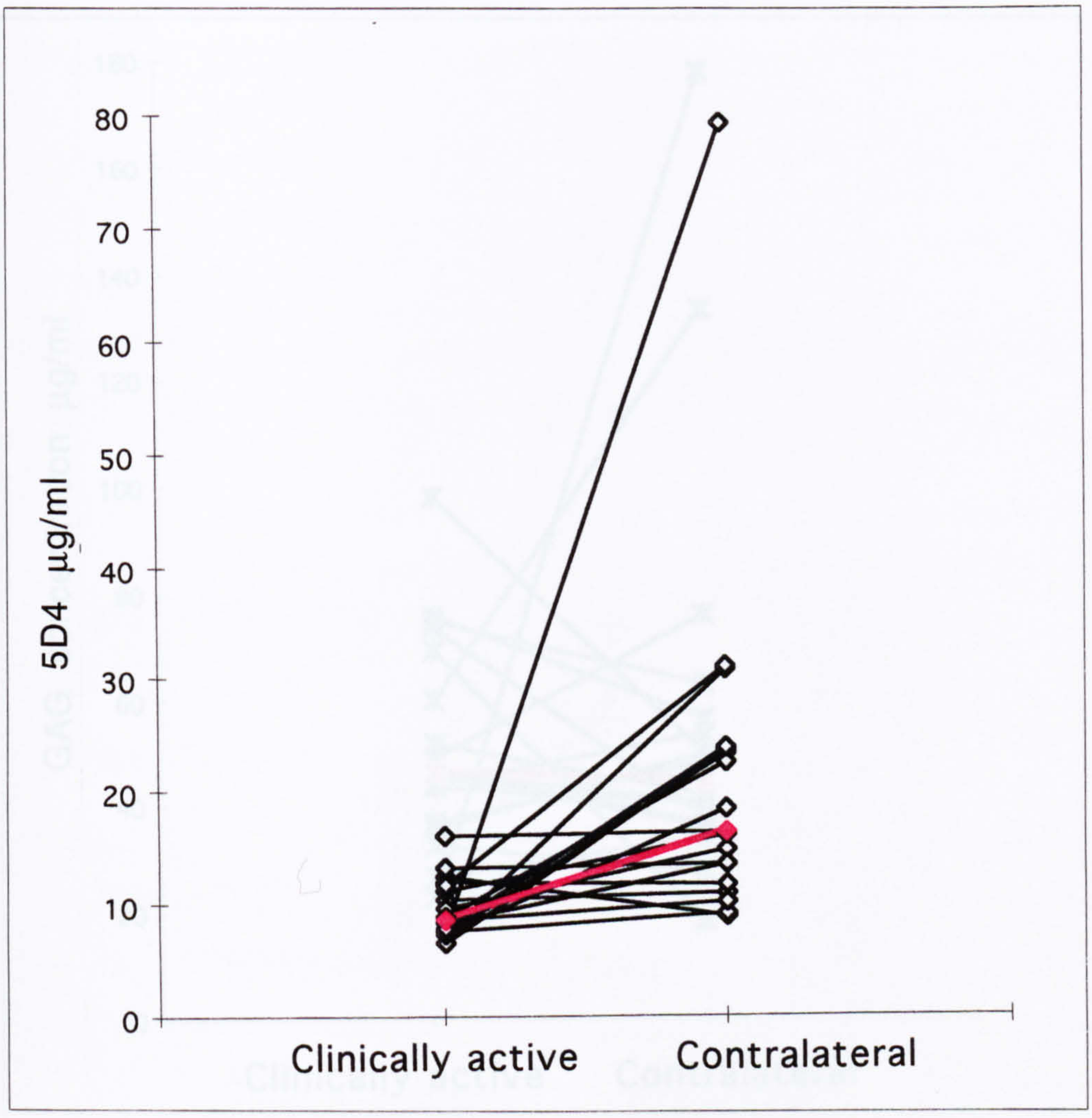

Figure 8-3: Cross sectional comparison of KS in matched joints.

The red line indicated the median values.

The median KS(5D4) concentration in clinically active joints was 8.79 (1.96) µg/ml, while the median KS concentration in the contralateral joint was 16.39 (5.65) µg/ml. This was a significant increase from the clinically active to the contralateral joint, $p = 0.001$ (Wilcoxon signed rank).

d) *Total glycosaminoglycans*

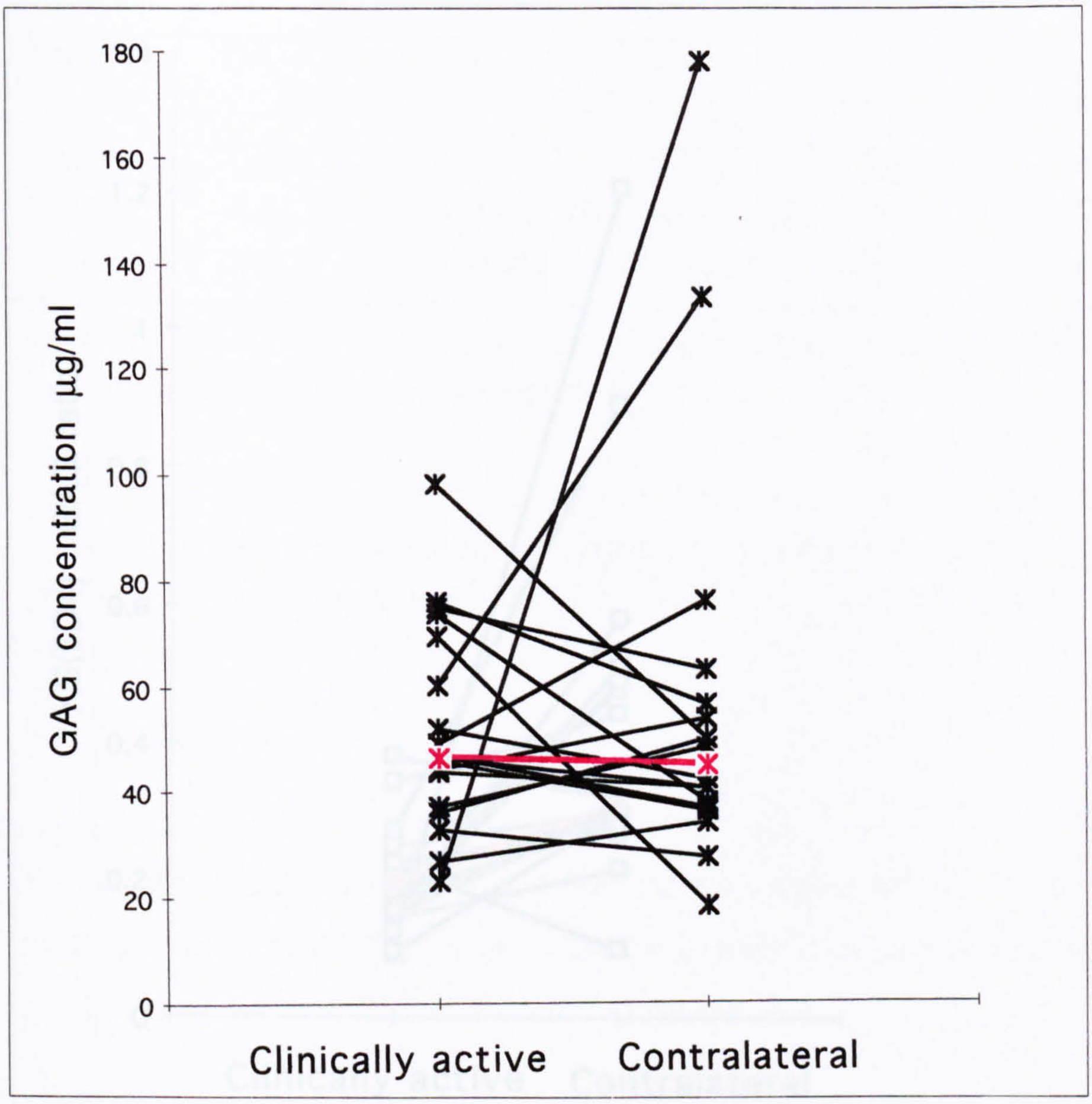

Figure 8-4: Comparison of GAG concentration in paired clinically active OA and contralateral joints.

The red line indicates the median values.

The median GAG concentration in clinically active joints was 46.85 (14.24) µg/ml while the median concentration in contralateral joints was 45.22 (9.21) µg/ml. There was no significant difference between paired joints.

e) *KS:GAG Ratio*

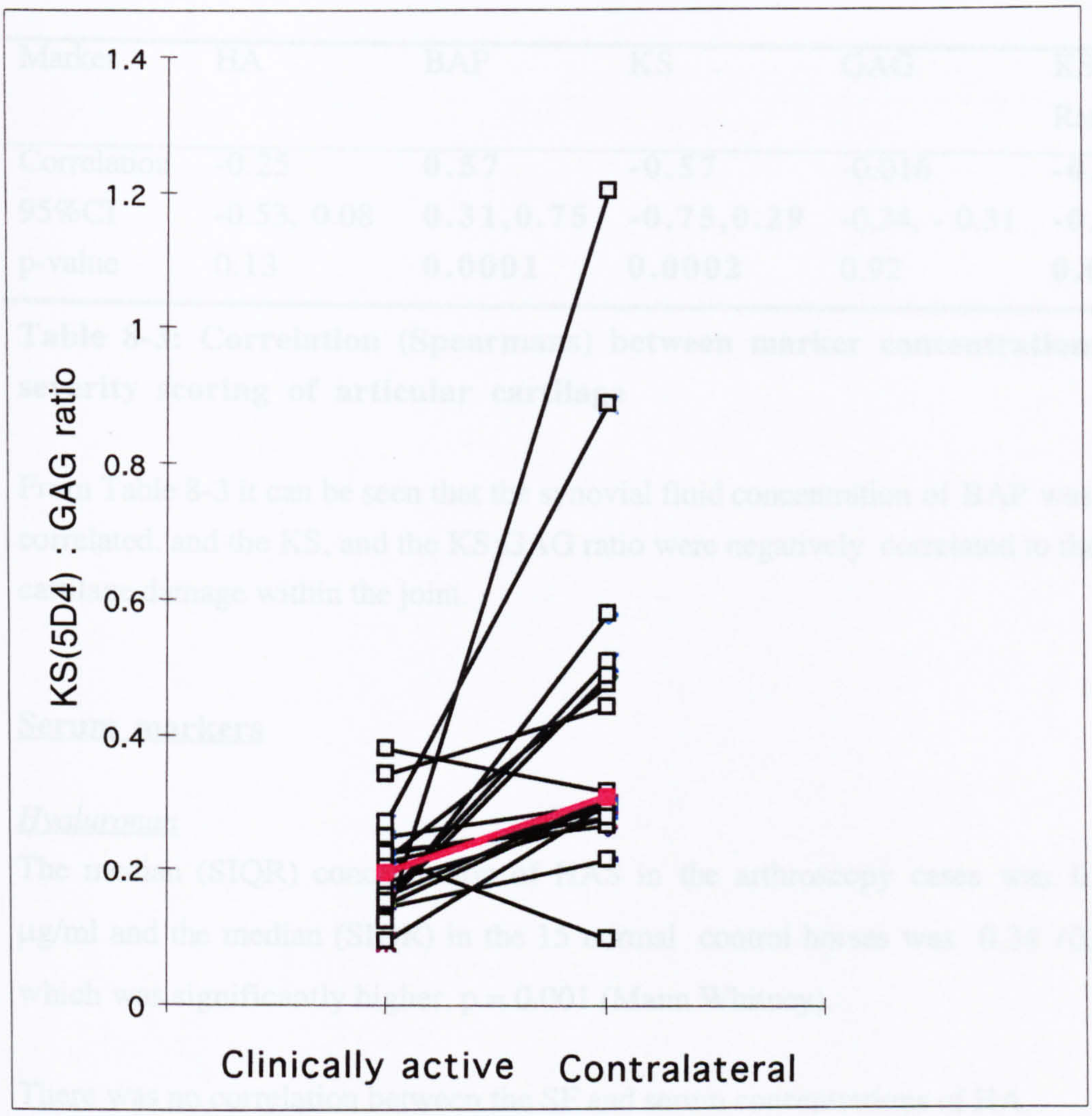

Figure 8-5: Comparison of KS:GAG ratio in paired clinically active OA and contralateral joints.

The red line indicated the median values.

There was a significant increase in KS:GAG ratio from the clinically active OA joints , in which the median ratio (SIQR) = 0.198 (0.04) to the contralateral, median = 0.309 (0.102), p=0.002 (Wilcoxon signed rank)

There was no correlation between the clinically active and the contralateral joint for concentration of any marker.

Correlation of synovial fluid markers with cartilage damage

Marker	HA	BAP	KS -	GAG	KS:GAG Ratio
Correlation	-0.25	**0.57**	**-0.57**	-0.016	**-0.49**
95%CI	-0.53, 0.08	**0.31,0.75**	**-0.75,0.29**	-0.34, - 0.31	**-0.7,0.19**
p-value	0.13	**0.0001**	**0.0002**	0.92	**0.002**

Table 8-3: Correlation (Spearmans) between marker concentrations and severity scoring of articular cartilage

From Table 8-3 it can be seen that the synovial fluid concentration of BAP was positively correlated, and the KS, and the KS:GAG ratio were negatively correlated to the degree of cartilage damage within the joint.

Serum markers

Hyaluronan

The median (SIQR) concentration of HAS in the arthroscopy cases was 0.18 (0.06) µg/ml and the median (SIQR) in the 15 normal control horses was 0.34 (0.05) µg/ml which was significantly higher, p = 0.001 (Mann Whitney).

There was no correlation between the SF and serum concentrations of HA.
There was no correlation between HAS and the arthroscopy score for active or contralateral joints.

2. OA cases

Case No	Age (years)	Sex	Breed	Duration (months)	Joint
1	15	M	TBX	12	MCP
2	12	M	H	12	FP
3	4	M	?	3	MCP
4	7	M	TB	3	MCP
5	4	F	TB	2	FP
6	10	M	H	5	DIP
7	9	M	TB	6	MCP
8	7	M	TB	2	MCP
9	14	M	H	7	TMT
10	9	F	H	4	TMT
11	9	M	TB	8	TMT
12	5	M	TB	3	MC
13	7	M	LAT	6	TMT
14	?	F	WBD	5	PIP
15	12	M	TB	3	MCP
16	9	M	WBD	8	TC
19	12	F	TBX	12	MC
20	13	M	ID	7	MCP
21	17	M	TBX	6	DIP
25	13	M	WBD	1	MCP
26	5	F	TB	5	TMT
29	8	M	TB	19	MCP
30	5	F	Arab	8	PIP
33	13	M	WCob	3	DIT
35	9	F	TBX	4	ABC
36	3	M	IDTB	1.5	GH
37	11	M	IDX	1	TC
39	16	M	TB	3	DIP
41	9	F	Arab	9	CMC

Table 8-4: Demographic variables of clinical OA cases

Abbreviations:
TB = thoroughbred, TBX = Thoroughbred Cross, ID = Irish Draught, H = hunter, WCob = Welsh Cob, WBD = warmblood, LAT = Latvian
MCP = metacarpophalangeal joint, PIP = proximal interphalangeal joint, DIP = distal interphalangeal joint, TMT = tarsometatarsal joint, DIT = distal intertarsal joint, ABC = antebrachiocarpal joint, MC = midcarpal joint, CMC = carpometacarpal joint, TC = tarsocrural joint, FP = femoropatellar joint, GH =glenohumeral joint.

From referred lameness cases 29 osteoarthritic horses were diagnosed (Table 8-4). The mean age of this group was 9.5 years (range 3 - 16 years) and the mean duration lameness was 5.7 months (range = 1 - 19 months). These horses were of mixed breeds, but 15/29 were Thoroughbred or Thoroughbred cross. Joints affected were also mixed, including 9/29 MCP, 5/29 TMT and 3/29 DIP joints. In most cases synovial fluid samples were only available from the clinically active OA joint. The results from these cases were combined with the baseline data from the CaPPS cohort for cross-sectional analysis. This combination resulted in a total number of 44 cases, of median age 9 years and median duration of lameness 4.5 months .

The concentrations of HAS, and HASF, KS(5D4), GAG and BAP were measured. The median HAS in this group was 0.32 (0.10) µg/ml. The SF marker results are shown in Table 8-5.

Marker		All joints	MCP	PIP	DIP	TMT	MC
KS µg/ml	Median	18.43	15.38	28.82	26.36	13.3	18.17
	SIQR	(8.01)	(3.62)	(10.05)	(9.17)	(7.19)	(4.25)
	n	41	10	5	9	8	3
	Range	1.14 - 69.23	3.58 - 25.11	4.52 - 69.23	7.07 - 49.28	1.14 - 34.71	9.69 - 26.71
GAG µg/ml	Median	56.08	46.56	121.76	72.36	48.69	46.14
	SIQR	(21.66)	(16.78)	(20.14)	(26.74)	(17.10)	(15.81)
	n	40	10	5	9	8	3
	Range	3.32 - 182.37	32.75 - 79.43	9.34 - 182.37	3.32 - 121.62	7.34 - 83.54	3.41 - 66.67
BAP Units	Median	8.53	5.51	20.64	5.58	10.07	9.12
	SIQR	(4.26)	(2.54)	(9.47)	(2.88)	(5.14)	(2.13)
	n	32	5	5	9	7	3
	Range	0.65 - 28.46	3.36 - 14.77	3.87 - 26.87	0.65 -, 13.97	9.63 - 28.46	4.83 - 13.35
HA- SF µg/ml	Median	261.5	1040	179	141	40.77	1204
	SIQR	(407.47)	(609.43)	(87.49)	(26.79)	(40.14)	(165.85)
	n	42	10	5	9	8	3
	Range	6.2 - 1972	272 - 1972	36 - 1934	74.3 - 203	6.2 - 139.9	701.6 - 1365

Table 8-5: Combined baseline SF marker concentrations from CaPPS and OA cases

Comparison of synovial fluid marker levels between OA cases and normals.

Comparisons between OA and normal data for HA, KS, and GAG SF levels were made within joint types since it was already known (Chapter 7) that these normal marker levels varied between joints. Since there were no joint differences in BAP levels, results for this marker from different joints were pooled for comparison.

a) Hyaluronan

The differences between HASF concentration in the MCP and PIP joints were not significant here, but the HASF concentration in the DIP joints was significantly lower than in the normal joints from this group of horses - $p = 0.0001$ (Mann Whitney) (Figure 8-6)

The difference between these results and those from paired joints in the arthroscopy group might be because the population in the OA group was more variable. Despite the lack of significance the trend was similar. The low numbers of PIP joints affected in the OA group may also have affected the comparison.

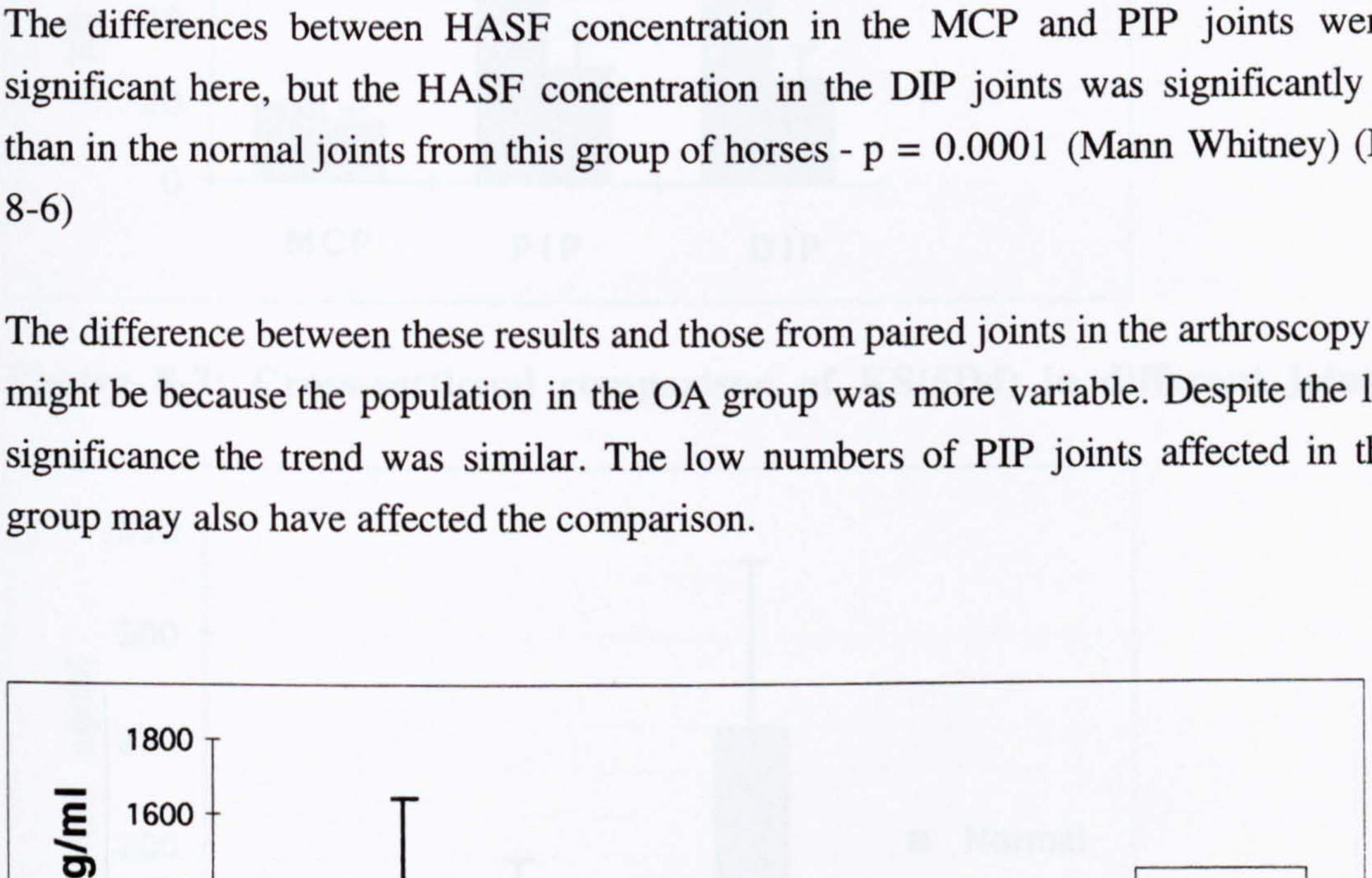

Figure 8-6: Comparison of normal and OA HASF values in different joints

b) KS and GAG

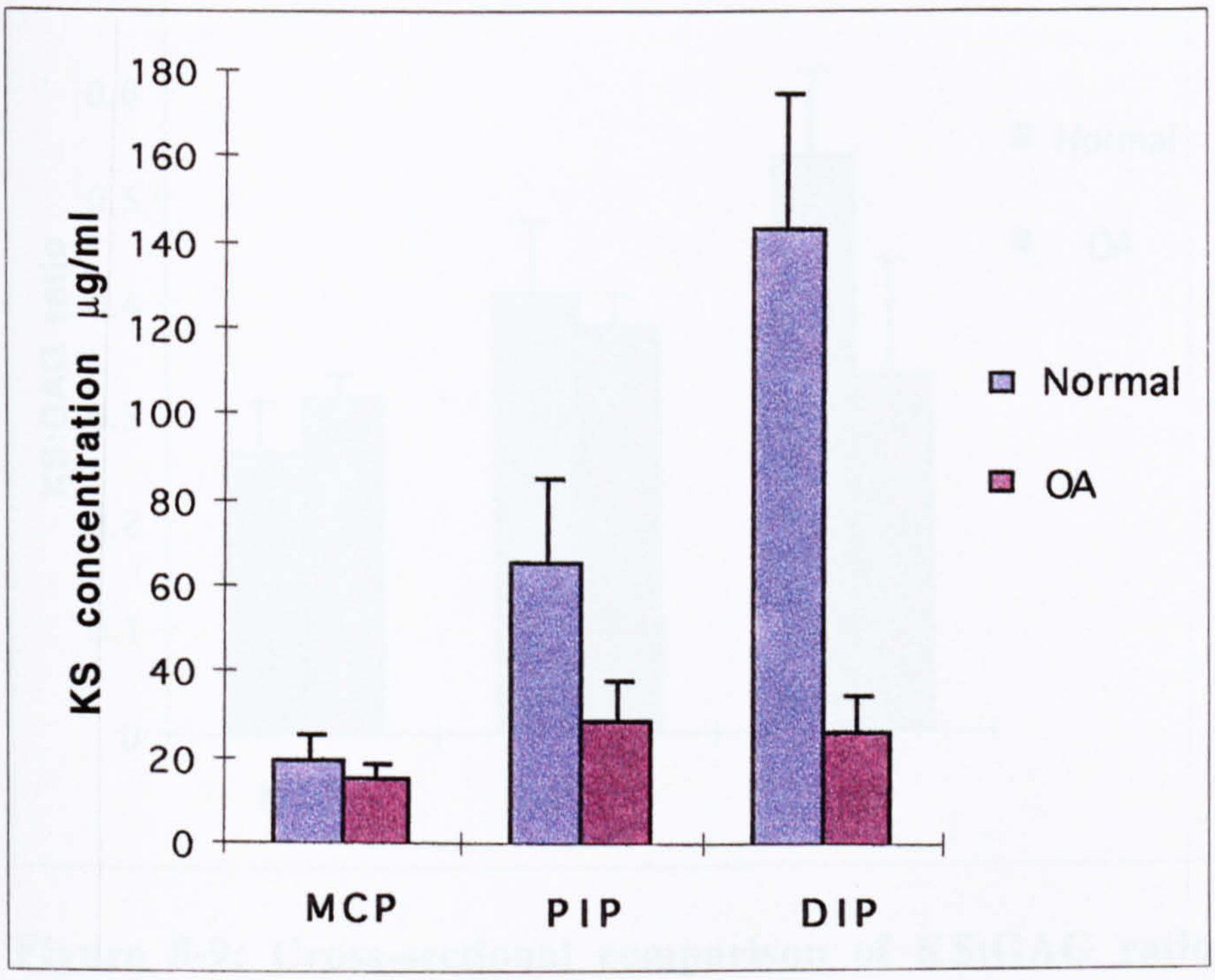

Figure 8-7: Cross-sectional comparison of KS(5D4) in different joints

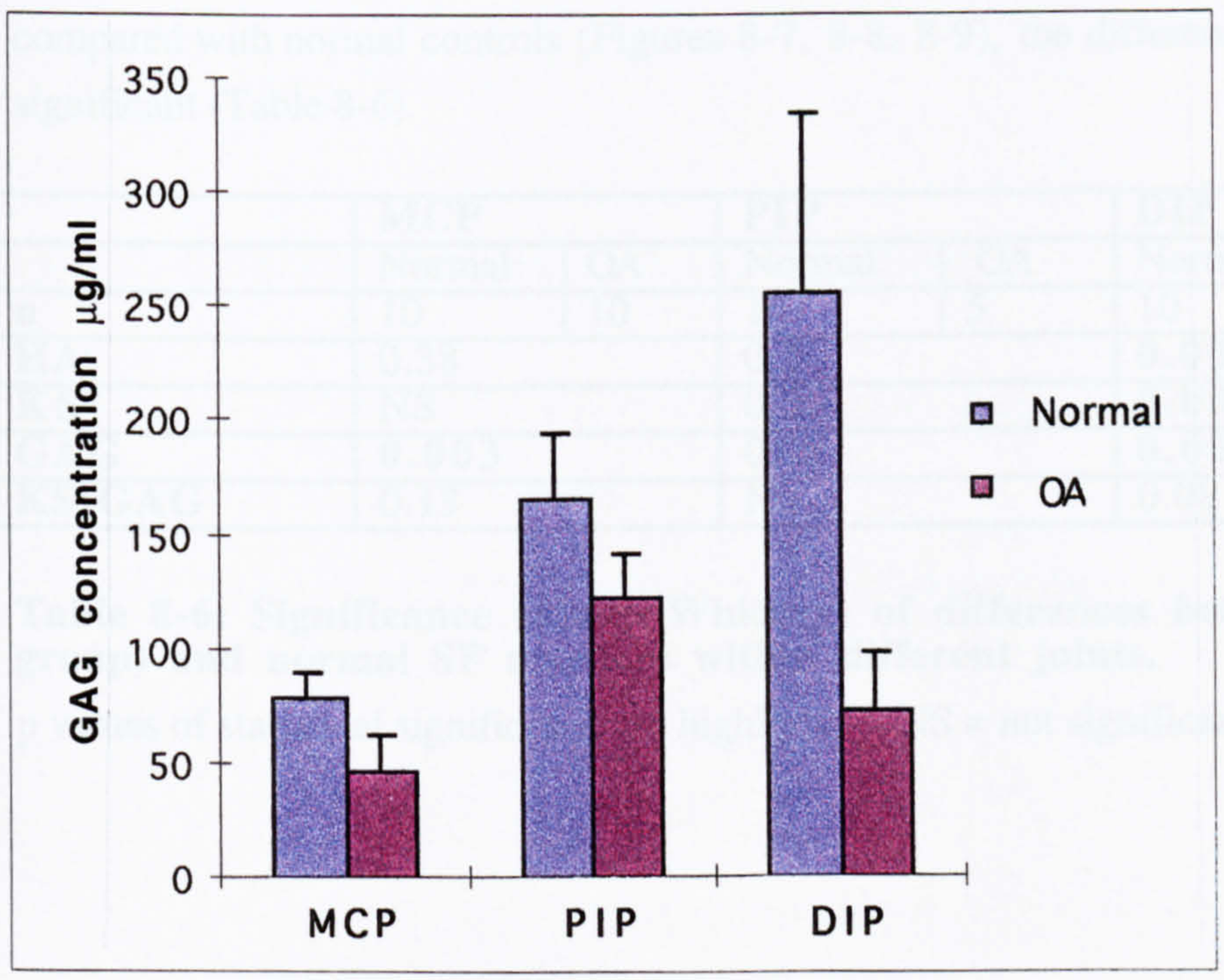

Figure 8-8: Cross-sectional comparison of SF GAG in different joints

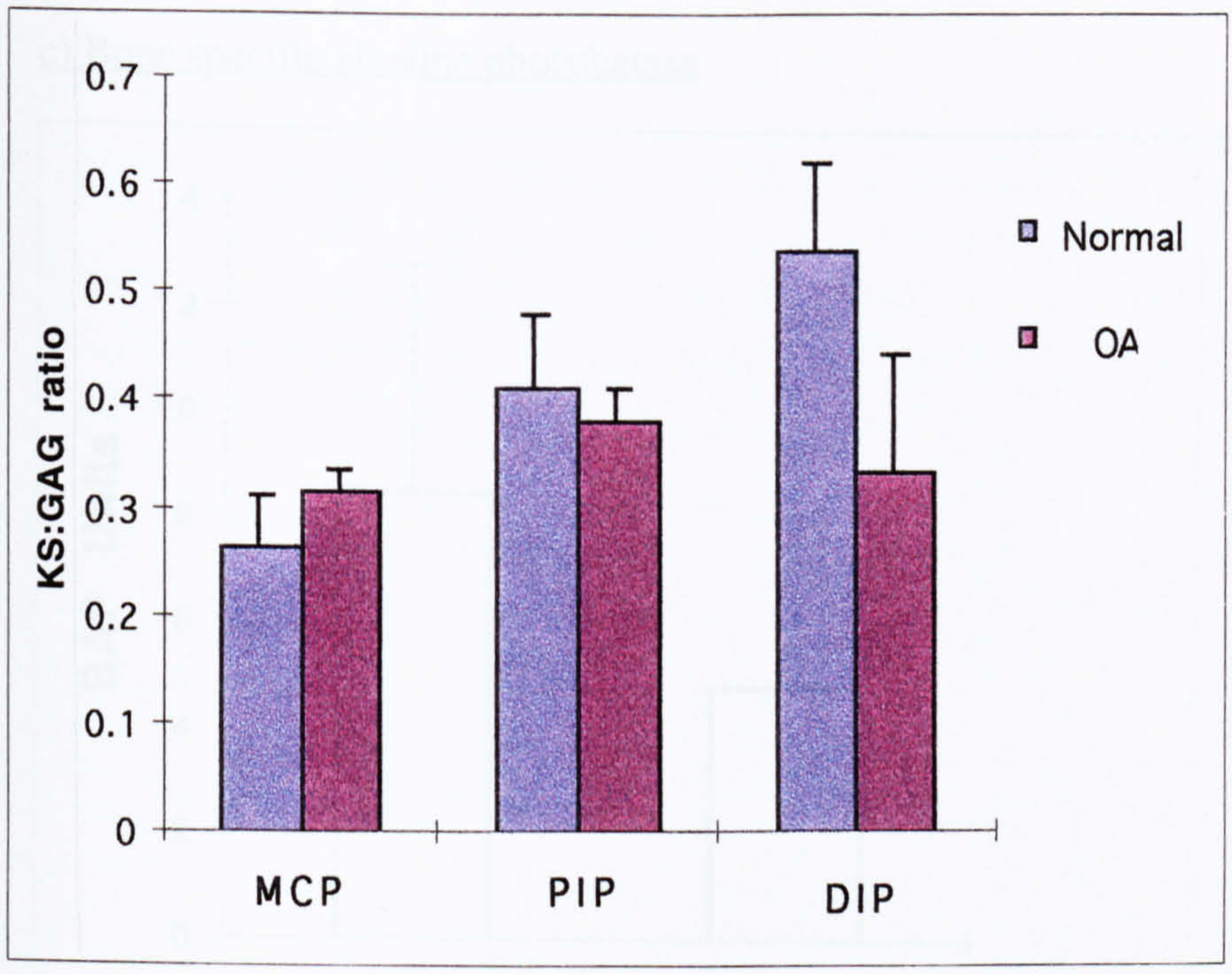

Figure 8-9: Cross-sectional comparison of KS:GAG ratio in different joints

Although the concentrations of KS and GAG tended to be lower in each OA joint when compared with normal controls (Figures 8-7, 8-8. 8-9), the differences were not always significant (Table 8-6).

	MCP		PIP		DIP	
	Normal	OA	Normal	OA	Normal	OA
n	10	10	10	5	10	9
HA	0.38		0.5		**0.0001**	
KS	NS		0.06		**0.0002**	
GAG	**0.003**		0.05		**0.0002**	
KS:GAG	0.13		NS		0.08	

Table 8-6: Significance (Mann Whitney) of differences between OA (in OA group) and normal SF markers within different joints.

p values of statistical significance are highlighted. NS = not significant

c) Bone specific alkaline phosphatase

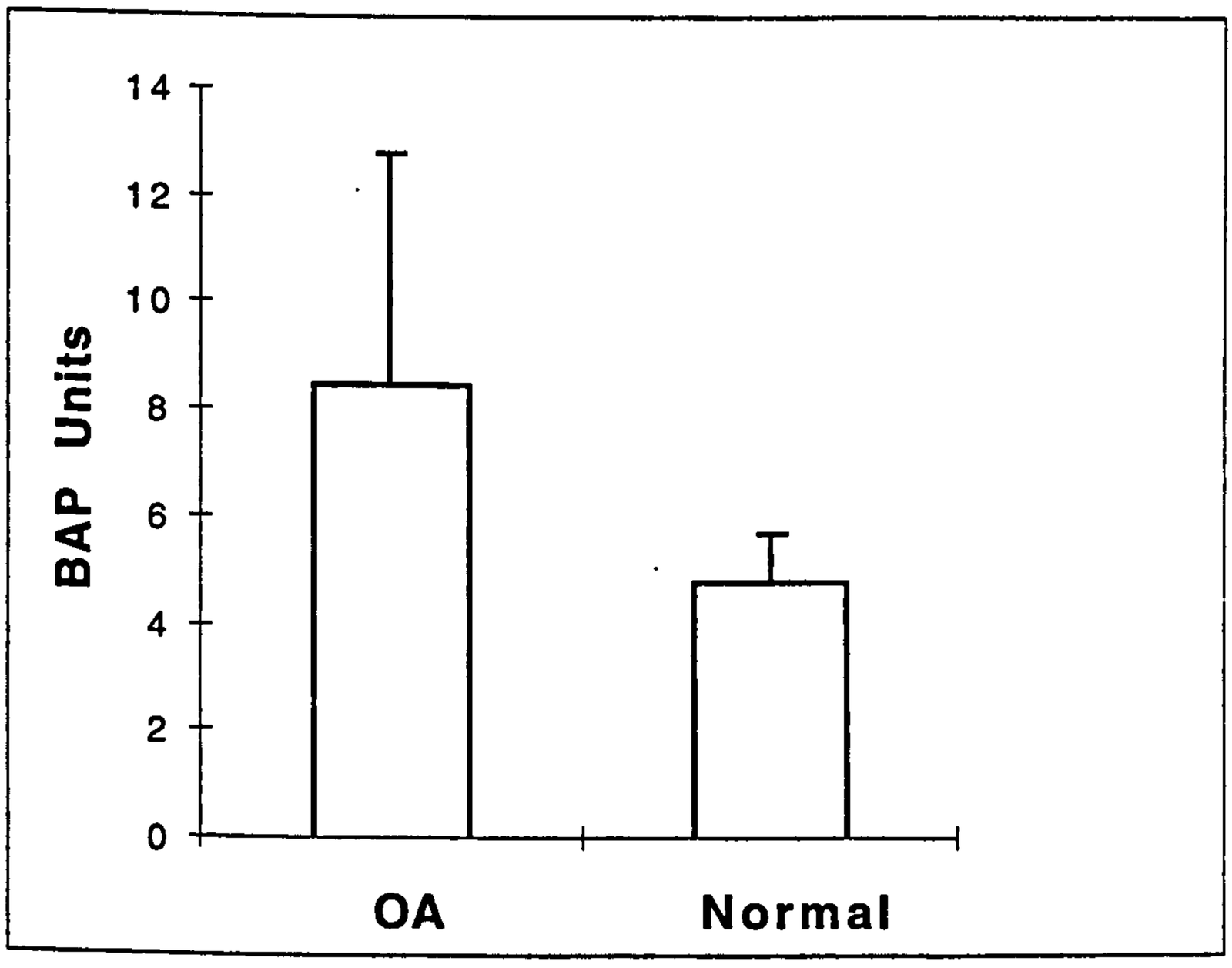

Figure 8-10: Cross-sectional comparison of BAP in OA and normal joints

There was a significant increase between BAP in the SF normal controls and OA joints , $p = 0.002$ (Mann Whitney).

Cross sectional comparison of HA in serum

The median HAS in the OA group was 0.32 µg/ml compared to a median concentration of 1.54 µg/ml in the normal controls.

This difference was very significant, $p < 0.0001$(Mann Whitney).

Correlation between markers and background variables

Marker concentrations in active OA joints and serum were compared with background variables i.e. age and duration of OA. The only significant correlations were between baseline value of KS in SF and age, i.e.

$r_s = 0.41$ 95%CI = 0.1 - 0.64 p = 0.009

and between baseline HAS and age, i.e.

$r_s = 0.45$ 95%CI = 0.048 - 0.73 p = 0.026

Difference between arthroscopy and OA populations

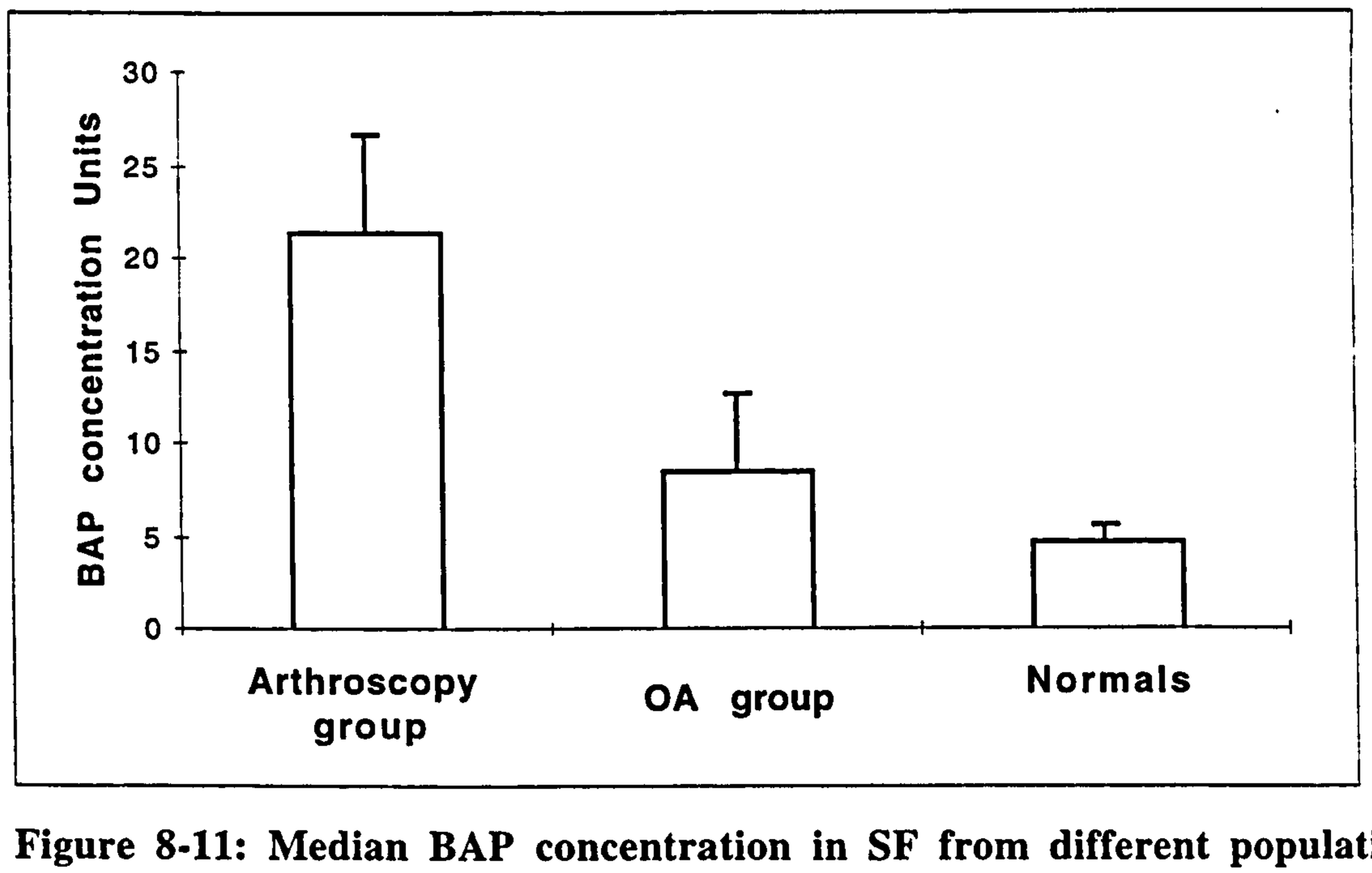

Figure 8-11: Median BAP concentration in SF from different populations.

Results indicated a difference between the arthroscopy and OA populations when compared to the normal controls (Fig 8-11). For this reason the overall levels of other markers measured were compared between these groups to see if a similar pattern was followed (Table 8-7).

	Arthroscopy (MC, ABC, MCP) active joints	Arthroscopy contralateral controls	OA (MCP, PIP, DIP joints only)	Normal controls (MCP, PIP, DIP) joints
Mean age	3.6 years	3.6 years	9 years	17 years
Median HASF	745.9 (249) n=17	1061 (325) n=17	261.5 (552) n=24	966.5 (311) n= 30
Within groups	**p = 0.01**		**p = 0.005**	
Between groups			* p = 0.09	**p = NS
Median BAP	21.75 (6.22) n-18	12.35 (4.31) n=18	8.52 n=32	4.77 n = 30
Within groups	**p = 0.002**		**p = 0.002**	
Between groups			***p = 0.0004**	****p=<0.0001**
Median KS(5D4)	8.79 (1.96) n=18	16.39 (5.65) n=18	20.39 (7.62) n=24	70.45 (53.69) n=33
Within groups	**p = 0.001**		**p = 0.0001**	
Between groups			***p = 0.0002**	****p = 0.0001**
Median GAG	46.86 (14.24) n=18	45.22 (9.21) n=18	68.65 (26.07) n=24	165.99 (78.65) n=33
Within groups	p = NS		**p = 0.0001**	
Between groups			*p = 0.28	****p = <0.0001**
Median KS:GAG	0.198 (0.04) n=18	0.309 (0.102) n=18	0.327(0.05) n=24	0.408 (0.113) n=33
Within groups	**p = 0.002**		p = 0.25	
Between groups			***p < 0.0001**	**p = 0.25

Table 8-7: Significance of differences between arthroscopy, OA, and normal populations for all markers (Mann Whitney).

p values of statistical significance are highlighted .

* = significance between active joints in different groups

** = significance of difference between normal controls in each group

From Table 8-7 it can be seen that there were significant differences between the arthroscopy and OA groups for levels of KS (5D4) as well as BAP concentrations in active and normal control joints. It is also interesting to note that when the results from the different joints are grouped together, the differences between the OA and normal controls are significant for every marker.

Differences in serum between arthroscopy and OA populations.

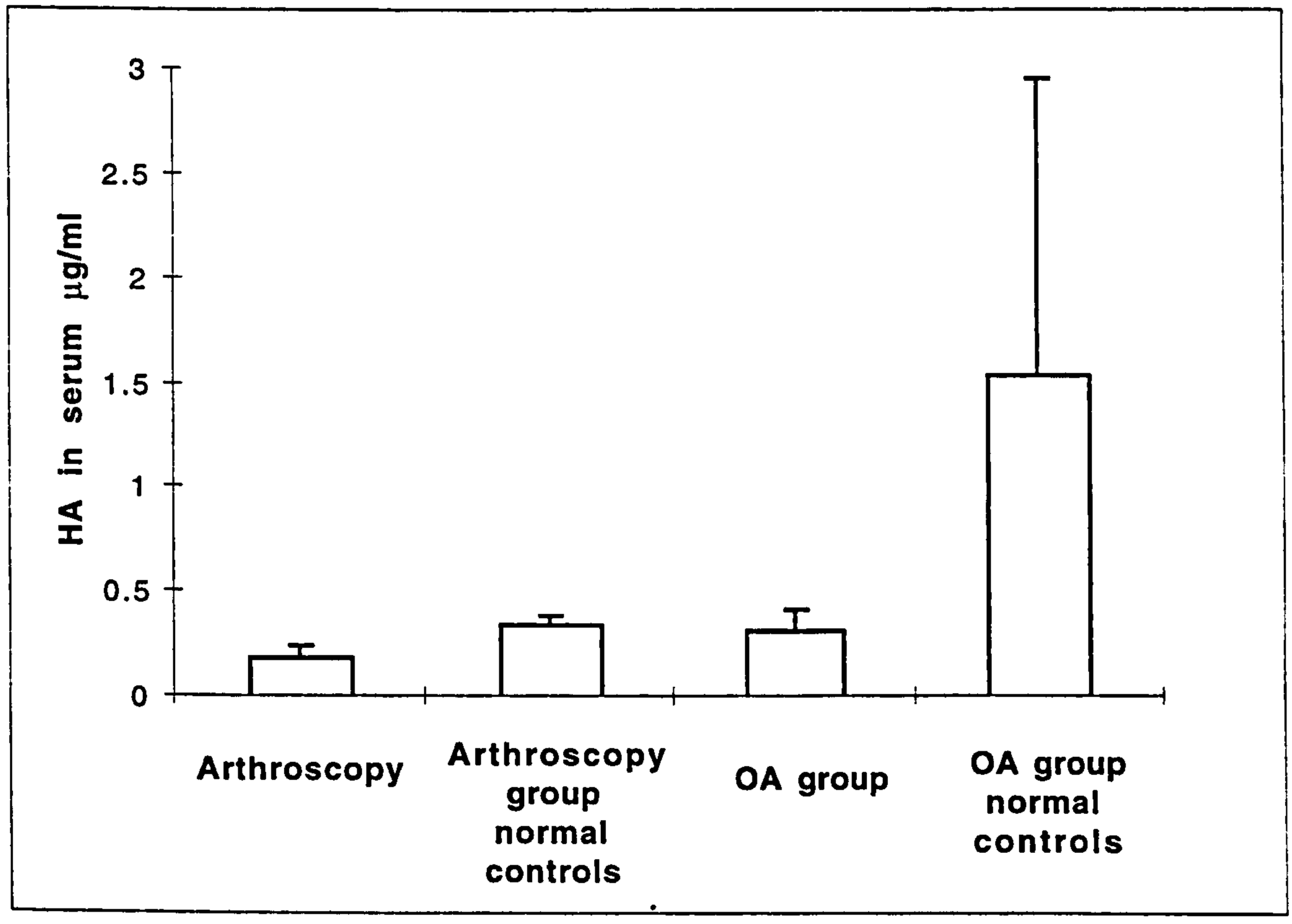

Figure 8-12: Cross-sectional comparison of HAS from normal, OA and arthroscopy cases

There were significant differences between HAS in normal, arthroscopy and OA cases (Table 8-8)

	Arthroscopy group n = 15	Normal controls Arthroscopy group n = 15	OA group n = 41	Normal controls for OA group n = 10
HAS µg/ml	0.18 (0.06)	0.35 (0.05)	0.32(0.10)	1.54 (1.41)
Within groups	p = 0.001		p < 0.0001	
Between groups	* p = 0.0006		** p < 0.0001	

Table 8-8: Median values and significance (Mann Whitney - U) between normal, arthroscopy and OA serum HA.

* = significance of difference between Arthroscopy and OA group
** = significance of difference between normal controls

Discussion

The clearly significant differences in HA, KS, KS:GAG ratio and BAP between active and contralateral joints in the arthroscopy samples indicate that these markers are useful in the cross-sectional assessment of OA in the horse. However the lack of significance between the OA and the normal controls in the OA group imply that without marker measurement in a paired inactive joint interpretation of results may be less clear.

The lower concentrations of HA and KS and the increased levels of BAP in the SF of the OA joint all agree with previously reported findings (Innes 1997; Todhunter *et al.* 1997; Tulamo *et al.* 1996) but the lack of significant difference in GAG levels does not (Alwan *et al.* 1991). Although differences between OA and normal joints were not always significant when compared within types of joint, the differences in marker levels in the OA joints followed the trends previously described. The lack of significance may have been caused by low numbers of some joints e.g. the PIP joint within the OA group, or by the differences in background variables between the OA joints and the normal control group used for comparison, in particular the median age for the OA and normal control group was unfortunately markedly different.

HAS was significantly altered in both the arthroscopy and OA groups when compared to that from normal horses. However, unlike the situation in man where HAS is raised in OA (Campion *et al.* 1991) in this study HAS was lowered. The reason for this is not

understood. However the use of HAS as a reliable marker of OA in the horse must be approached with caution. No correlation between HASF and HAS could be found. The wide variation in normal values of HAS between horses has been reported (Chapter 7), and HAS been shown to be affected by exercise and by age (Tulamo *et al.* 1996). In this study HAS was positively correlated with increasing age as was KS(5D4) in the SF. This has also been previously reported in human studies and is thought to be due to increased length and number of 5D4 epitopes on the KS chains.

The correlation between arthroscopic score of cartilage damage and marker levels imparts further validity to the use of SF BAP and KS (5D4) in cross-sectional OA assessment. As noted in the introduction to this chapter, correlation between decreasing levels of KS (5D4) in the SF and increasing degrees of cartilage damage has previously been reported. However the positive correlation between SF BAP and articular cartilage degeneration is an unreported finding and demonstrates a link between changes in bone and articular cartilage in OA.

Another interesting result from this study is the demonstration by marker measurement of clear differences between the arthroscopy and OA populations of horses. These two populations were chosen to provide cases of early and late stage OA , but no correlation between marker levels and duration of the disease was detected. Not only were there significant differences between levels of BAP and KS in the arthroscopy and OA group active joints, but the levels of these markers in the arthroscopy contralateral joints and OA group normal control joints was also very significant. HAS levels were also lower in arthroscopy normal control horses when compared with the OA group normal controls. Correlation between SF KS and HAS and age of horses may be relevant here. The horses undergoing arthroscopic examination and their normal controls were all young (mean age 3.6 years) while the OA group horses were older (mean age 9 years) and their controls still older (mean age 17 years). This may account for the increase in HAS in the OA control group when compared with the younger arthroscopy population. It may also be the reason behind the higher level of KS(5D4) in the OA and OA controls when compared with the arthroscopy group. However the BAP concentrations were also significantly higher in both the arthroscopy active joints and the contralaterals than in the OA joints and normal control joints. No correlation could be found between BAP concentrations and age, duration of OA or type of joint. The joints included in the analysis of marker levels in the OA group were MCP, PIP, and DIP joints, while the majority of joints examined in the arthroscopy group were MC joints. Although no differences in normal BAP concentrations were found in different joints, investigations of normal variations did not include MC joints.

It should be remembered that the horses undergoing arthroscopic examination were in training and it is possible that differences in the use of the horses as well as the breeds involved may have been responsible for the reported marker differences. In one human study differences in serum KS between soccer players and runners was demonstrated (Roos *et al.* 1995) which was thought to be the effect of the different physical activity. It may also be that the articular cartilage pathology in the arthroscopy horses was different to that in the OA cases and may have been better classified as "traumatic arthritis" rather than early OA. In 1996 Tulamo reported (Tulamo *et al.* 1996) that HASF was higher in horses with acute traumatic arthritis than in those with OA, and although HASF showed no significant difference between the groups in this study, the alterations in the other markers could be because of a difference in the disease pathology. Synovial effusion was more common in the MC joints of the arthroscopy group than in the joints in OA group and this may have reduced concentrations of KS, but would have been unlikely to effect the KS:GAG ratio , or been the cause of the increase in the BAP concentration or alterations in the serum markers. This finding illustrates the point that as well as their potential as diagnostic aids in OA, biochemical markers provide information concerning the pathological events occurring within the joint in OA which helps in the understanding of the course of the disease. A panel of markers provides more useful information than one marker used in isolation, and an extension to this study should include the statistical analysis of the relationship between the markers studied here with allowance for the correlation with background variables.

Summary

1. Concentrations of HASF, KS, KS:GAG, and BAP are all significantly different in OA compared to contralateral joints and are therefore useful as cross-sectional markers in OA.

2. BAP, KS and the KS:GAG ratio show good correlation with articular cartilage pathology, which imparts criterion validity to these markers.

3. HAS and KS (5D4) in SF are positively correlated with increasing age.

4. The use of markers here demonstrated a clear difference between two populations of horses.

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Chapter Nine

Biochemical markers in osteoarthritis :

Longitudinal studies and correlations

Introduction

The normal variations of synovial fluid (SF) and serum markers in osteoarthritis (OA) have been discussed in Chapters 7 and 8. In this chapter, temporal changes in the same markers will be described, in particular in relation to the use of these markers as measures of outcome as assessed in the Calcium pentosan polysulphate (CaPPS) trial.

The common goals of the many scientists researching the use of biochemical markers include the ability to diagnose OA at an earlier stage in disease than by the traditionally accepted diagnostic imaging techniques, the differentiation of early and late stages of the disease, and the prediction of subsequent OA progression. Many cross-sectional studies investigating the use of these markers as diagnostic tools have been carried out, both in man and animals, and the results from these are promising. However the aim of finding markers capable of assessing longitudinal changes in OA and predicting progression of the disease requires prospective temporal studies, and very few have been reported in the literature.

In 1994 Dahlberg and colleagues (Dahlberg *et al.* 1994) measured aggrecan, cartilage oligomeric matrix protein (COMP), stromelysin, and TIMP-1 in SF samples obtained from human knee joints, directly following injury, and again 6 months and then 6 years later. The results were compared with those from samples from normal healthy volunteers. They showed that all markers increased significantly immediately following injury, but then fell to levels similar to reference controls. These results were in contrast to those reported by Lohmander *et al*, also in 1994 (Lohmander *et al.* 1994), which showed that levels of stromelysin, TIMP and proteoglycan in the knee SF rose significantly during the first day following injury, and then remained higher than controls. In canine studies of stifle OA (Innes 1997) the 5D4 epitope of keratan sulphate (KS(5D4)) dropped significantly 6 weeks following stifle surgery. No significant longitudinal changes were detected in hyaluronan (HA) or bone specific alkaline phosphatase (BAP). In one report of longitudinal SF marker measurements in the horse (Todhunter *et al.*

1993) KS(5D4) was measured in experimentally induced OA in ponies and was found to peak within 24 hours of surgery, but then fell to, and remained at, normal reference values.

The use of biochemical markers has been explored as another potential method of outcome measurement in clinical trials, but as yet has not been validated for such use (Lequesne *et al.* 1994). Correlations between marker changes and other outcome measurements have frequently been made however in an attempt at validation. In 1996 Fawthrop (Fawthrop *et al.* 1997) compared marker levels in two groups of patients classified as having progressive or non-progressive knee OA defined by radiological changes over a 2 year time period. No significant differences were found between groups for levels of chondroitin sulphate (CS), KS, HA, or total glycosaminoglycans (GAG) in the SF. However in 1995, Sharif *et al* demonstrated the predictive value of both serum HA (HAS) (Sharif *et al.* 1995) and an increase in serum COMP (Sharif *et al.* 1995) in determining progressive OA of the knee, again classified by radiological change. HAS has also been found to correlate with knee radiography score and the general pattern of scintigraphic scan (Campion *et al.* 1991). Serum COMP has been found to correlate with disease stage in OA (Lohmander *et al.* 1994) and more recently with scintigraphy scores (Petersson *et al.* 1998). In dogs, serum GAG was found to correlate with scintimetry indices and BAP correlated with radiographic osteophyte score (Innes 1997). However in a study by Belcher (Belcher *et al.* 1997) no correlations could be detected between radiographic scores and KS(5D4), GAG HA or 3B3 levels in the SF in knee OA.

In order to validate a measure of outcome it should correlate with a known gold standard for the disease, which in the case of OA has traditionally been considered to be radiography. However in previous studies radiography has not proved to be a useful measure in chronic stages of OA and does not always correlate with other validated measures e.g. clinical changes (Dieppe *et al.* 1997). This may be because clinical and radiographic changes do not always occur in synchrony. In this study the clinical cohort were horses in various stages of chronic OA and the chosen gold standard measure of outcome was the global score of lameness improvement. Correlation of markers with this measure would therefore seem a more relevant method of validation .

It is clear from the diversity of results reviewed that more longitudinal studies need to be undertaken before the temporal changes occurring in early through to late stage OA and their relevance to OA can be understood. It is likely that it will be the altering relationship between a panel of markers, rather than changes in one marker in isolation , that will ultimately be identified as useful in assessing OA progression.

Aims

1. To investigate longitudinal changes in markers during the 9 month period of the trial.

2. To assess validity of markers by investigating correlations between marker changes and clinical outcome, scintigraphy, and radiography.

3. To investigate differences in outcome between the two treatment groups in the CaPPS trial.

Methods

The horses used for this study were those from the CaPPs trial. Only 14 horses had SF samples at sufficient time points for longitudinal analysis. For materials and methods see Chapter 4 for the trial design and Chapter 7 for marker assays.

Statistical analysis.

Horses were paired for analysis. Group comparisons of skewed data was by Wilcoxon matched pairs and of normally distributed data by Paired students t-test. Correlations were by Spearmans correlation coefficient where results were nonparametric i.e. SF samples and by the Pearsons correlation coefficient where results were parametric. Confidence intervals at 95% were stated and significance was accepted at 5%. For estimations of power see Chapter 4.

Low dose group - 0.5 mg/kg						High dose group - 2mg/kg					
Horse	**Sex**	**Age**	**Breed**	**Duration (months)**	**Joint**	**Horse**	**Sex**	**Age**	**Breed**	**Duration (months)**	**Joint**
1	M	11	IDX	6	TMT	5	M	21	Dales Pony	2	TMT
3	M	12	TB	2	PIP	14	M	11	TBX	1	PIP
7	M	13	Pony	4	MC	15	M	5	TBX	4	MCP
8	F	8	TB	3	DIP	9	F	9	ID	3	DIP
13	M	9	Hunter	24	TMT	6	F	7	TBX	24	TMT
20	M	14	Welsh Cob	5	DIP	19	F	18	TBX	5	DIP
16	M	8	Lusitano	1	DIP						
18	F	10	TB x ID	18	PIP						

Table 9-1: Demographic variables of horses included in longitudinal study showing pairing.
Horses 16 and 18 were not included in paired comparisons.

Results

Longitudinal studies

For the demographic variables see Table 9-1.

Longitudinal SF samples were available from 14/19 horses on the CaPPS trial. These were matched for analysis as shown in Table 9-1. Two horses in the low dose could not be matched (16 and 18), but data from these were included in baseline predictions and correlation analysis. There was no significant difference between the groups for age or duration of OA (Paired student t test).

Synovial fluid markers

a) Hyaluronan

There was no significant difference between HA in SF between the high and low dose treatment groups at any sampling point (Wilcoxon signed rank) (Figures 9-1a and b).

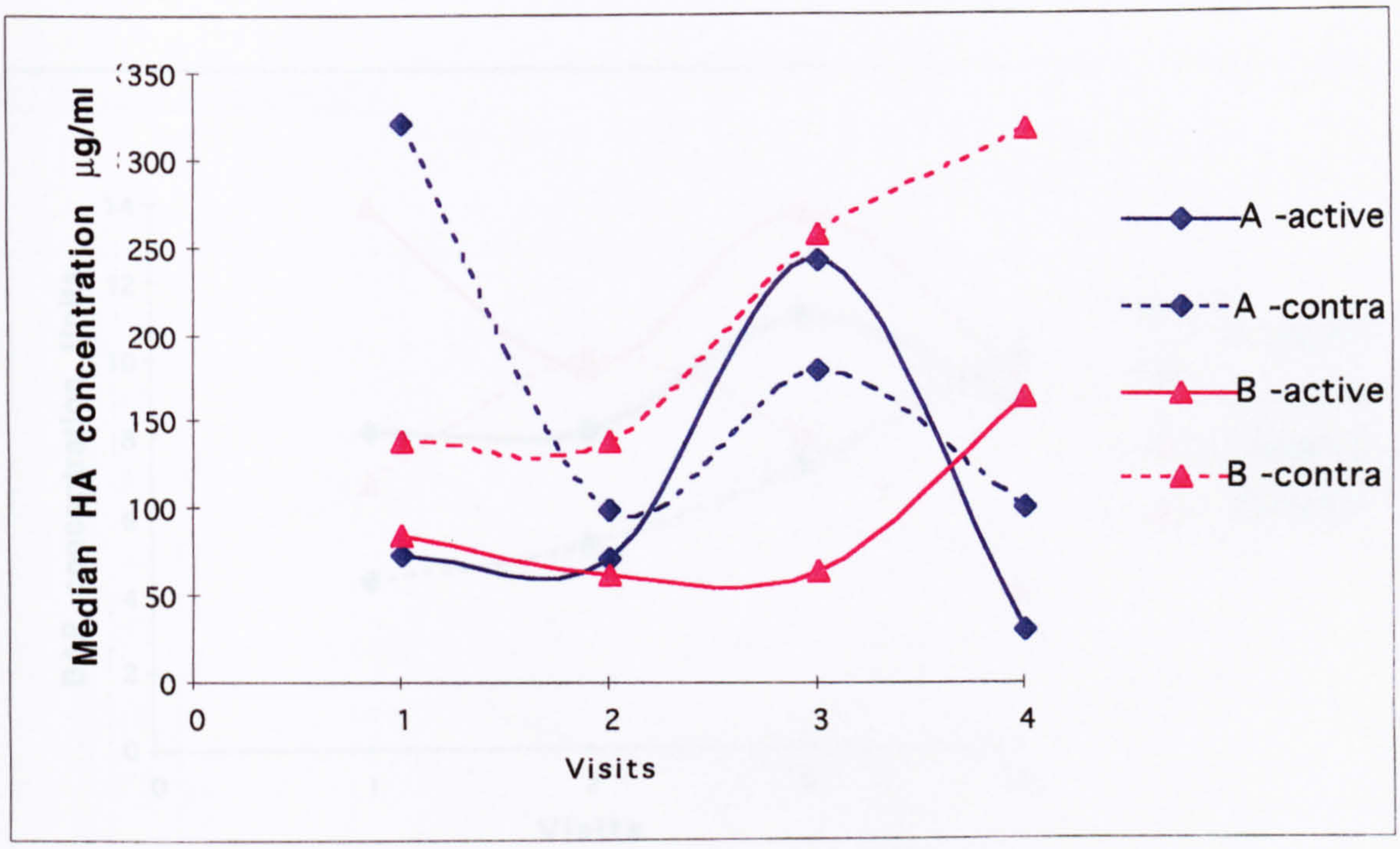

Figure 9-1a: Longitudinal variation in median HASF concentration in matched active and contralateral joints.

A = low dose group, B = high dose group.

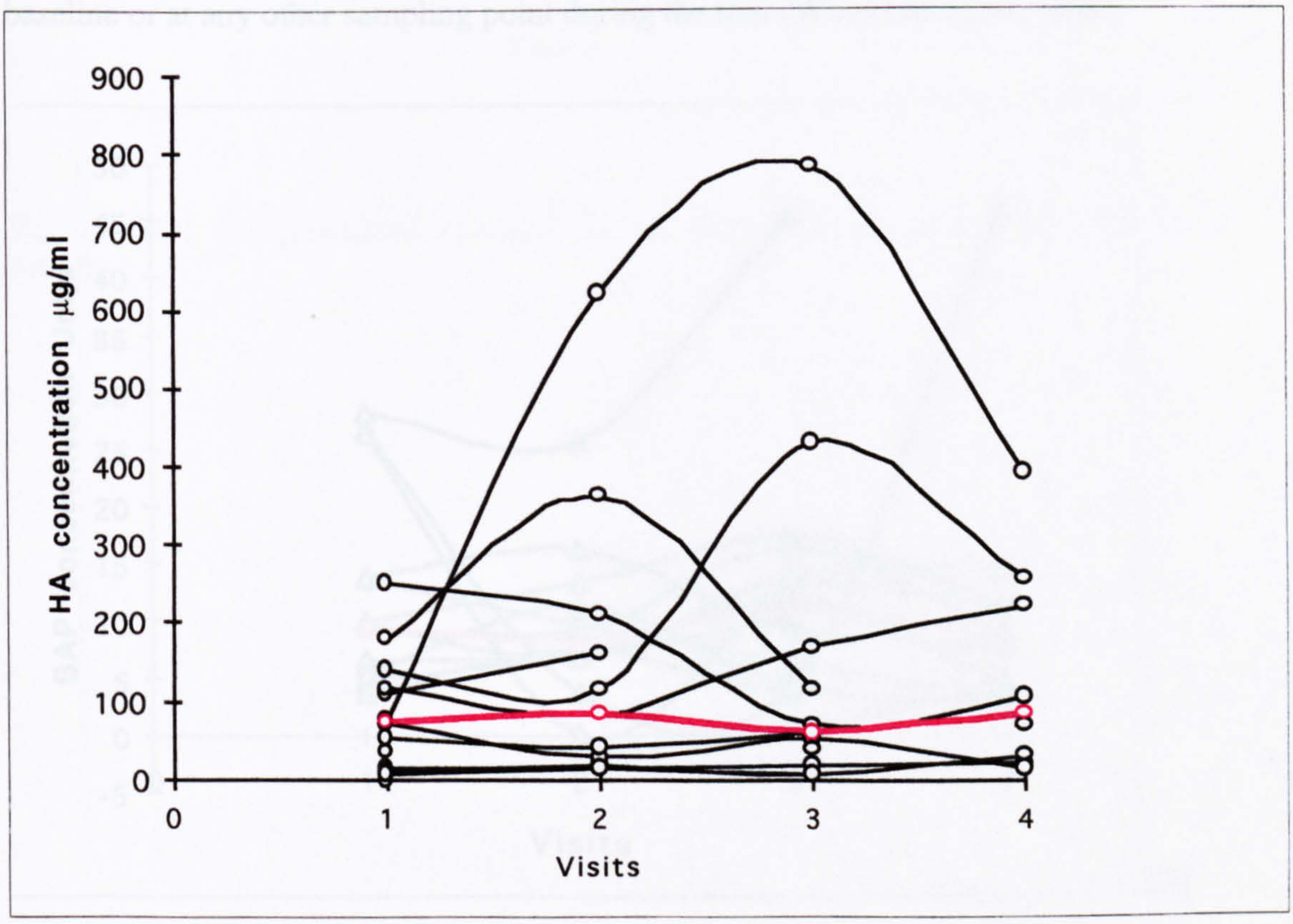

Figure 9-1b: Longitudinal HASF concentration for all horses

Median value = red line

b) Bone specific alkaline phosphatase

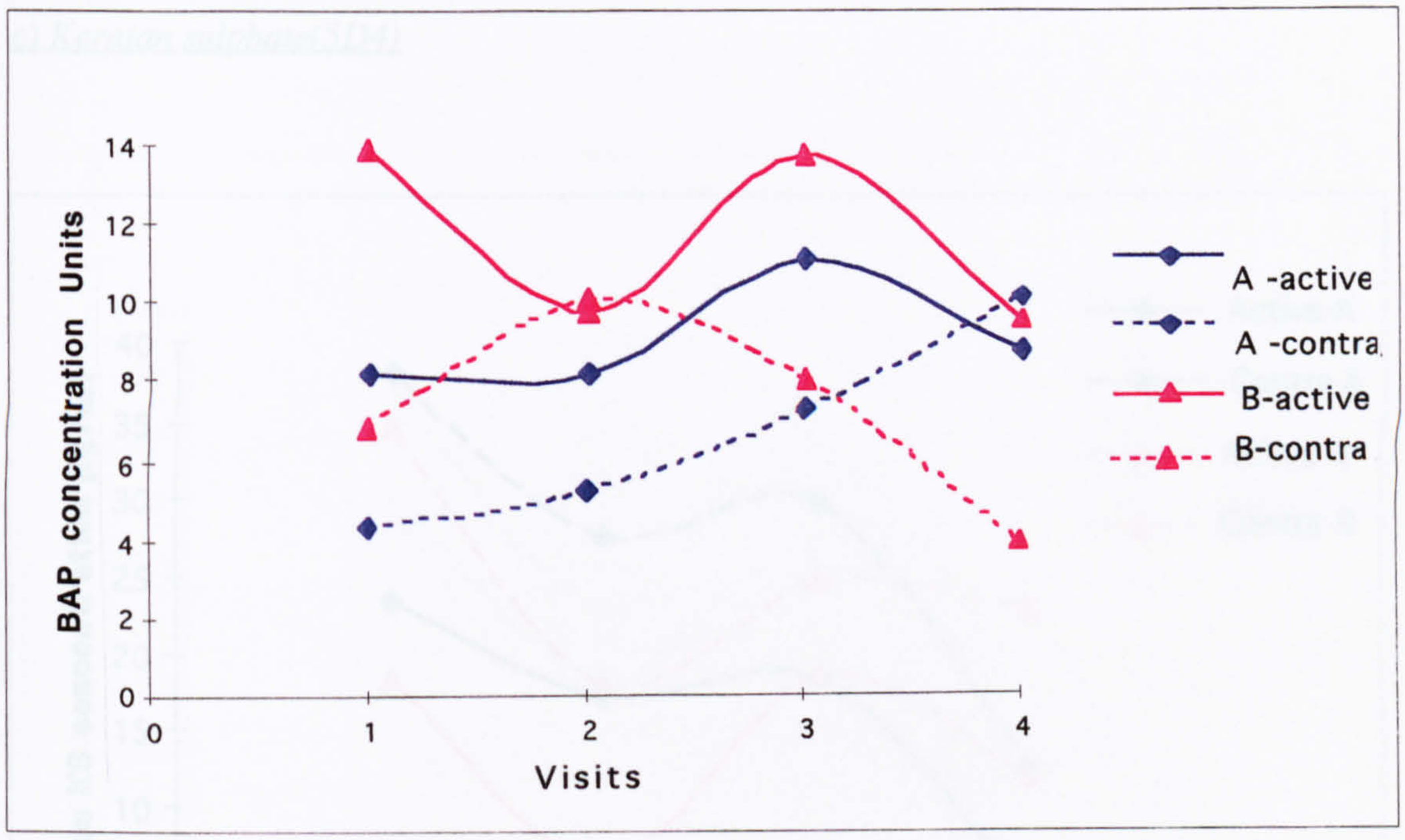

Figure 9-2a : Longitudinal changes in median BAP concentrations.

There was no significant difference between groups for BAP concentrations either at baseline or at any other sampling point during the trial (Wilcoxon signed rank).

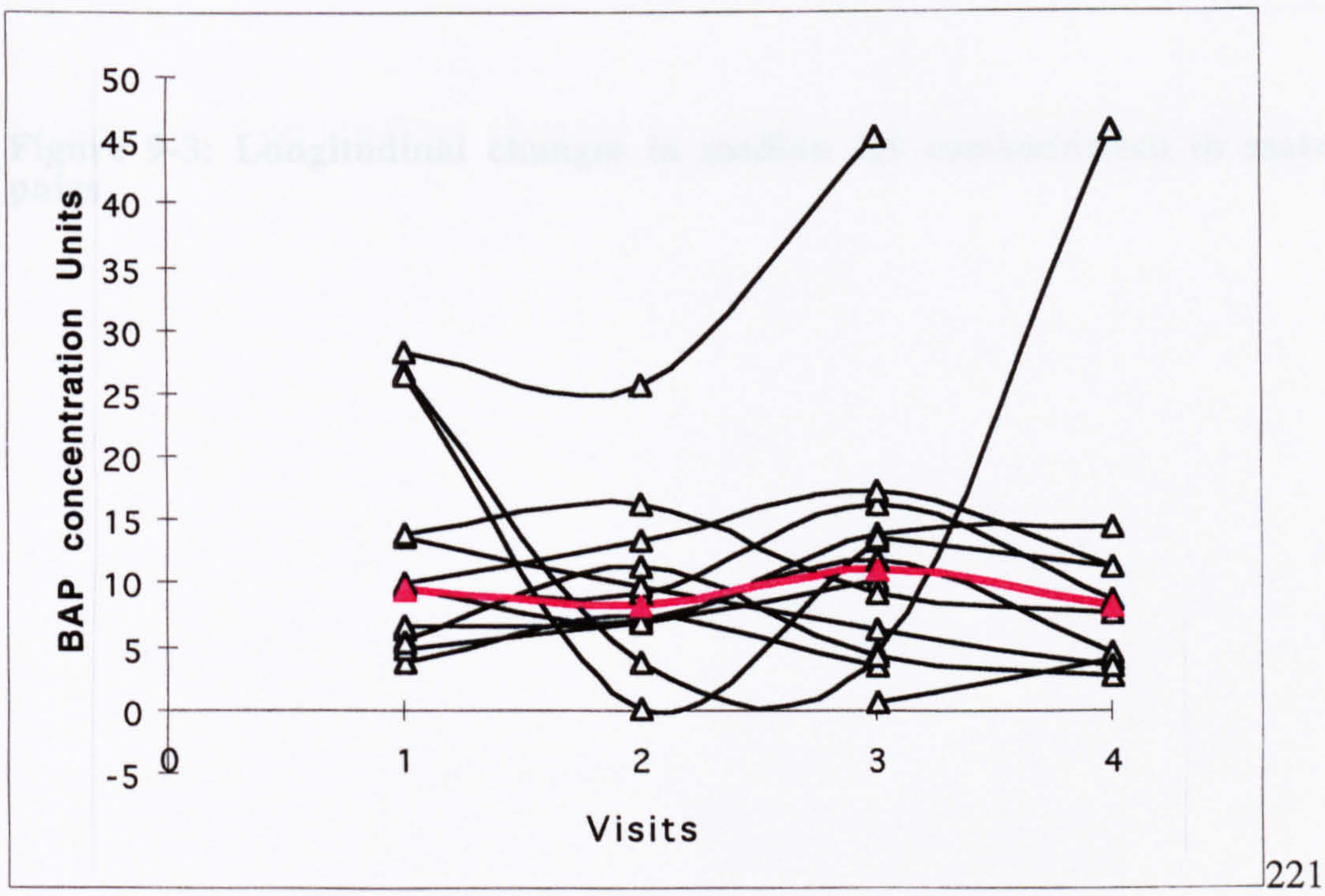

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Figure 9-2b: Longitudinal changes in BAP for all active joints.

Median value = red line

c) Keratan sulphate(5D4)

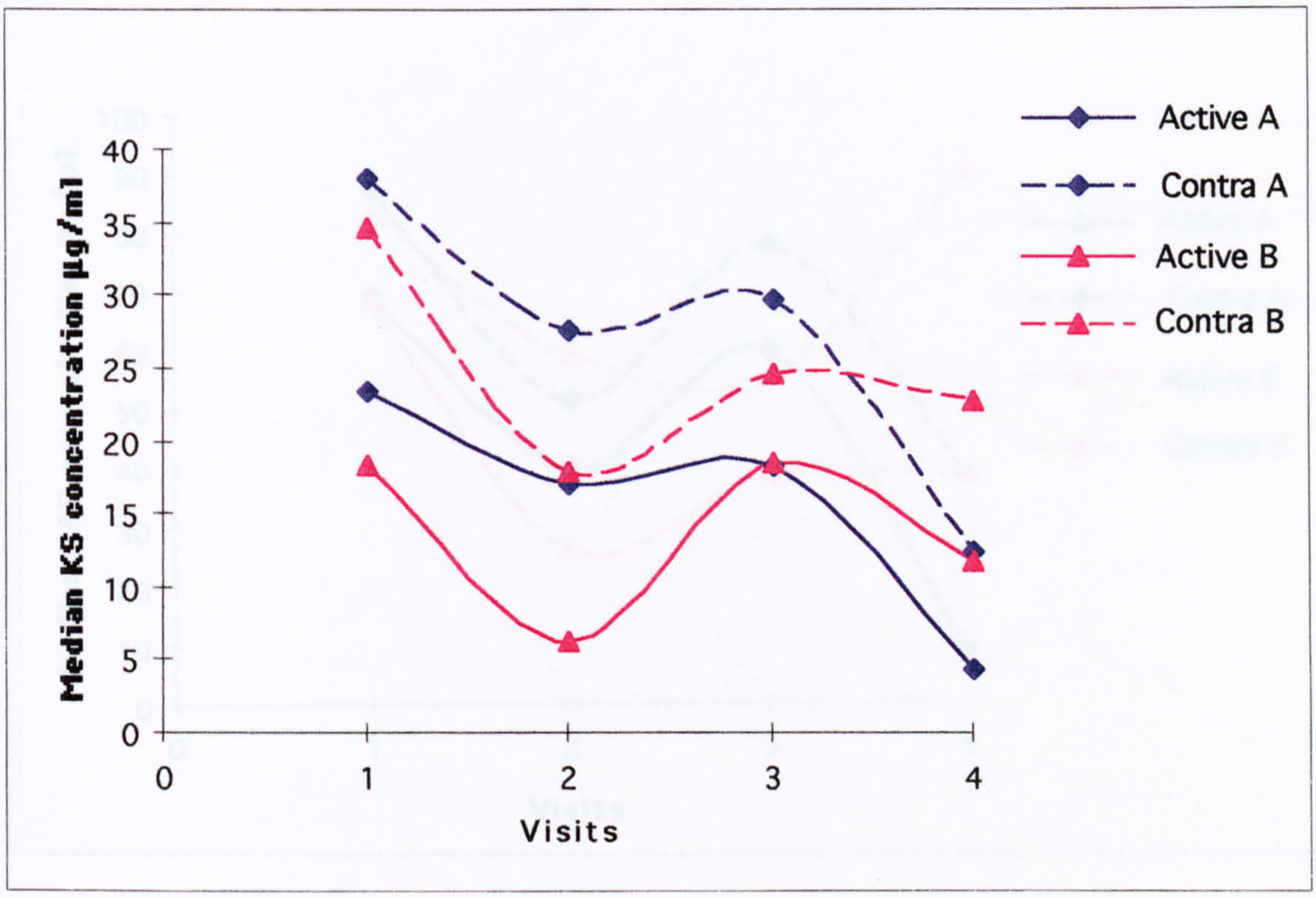

Figure 9-3: Longitudinal changes in median KS concentration in matched pairs

d) Total glycosaminoglycans

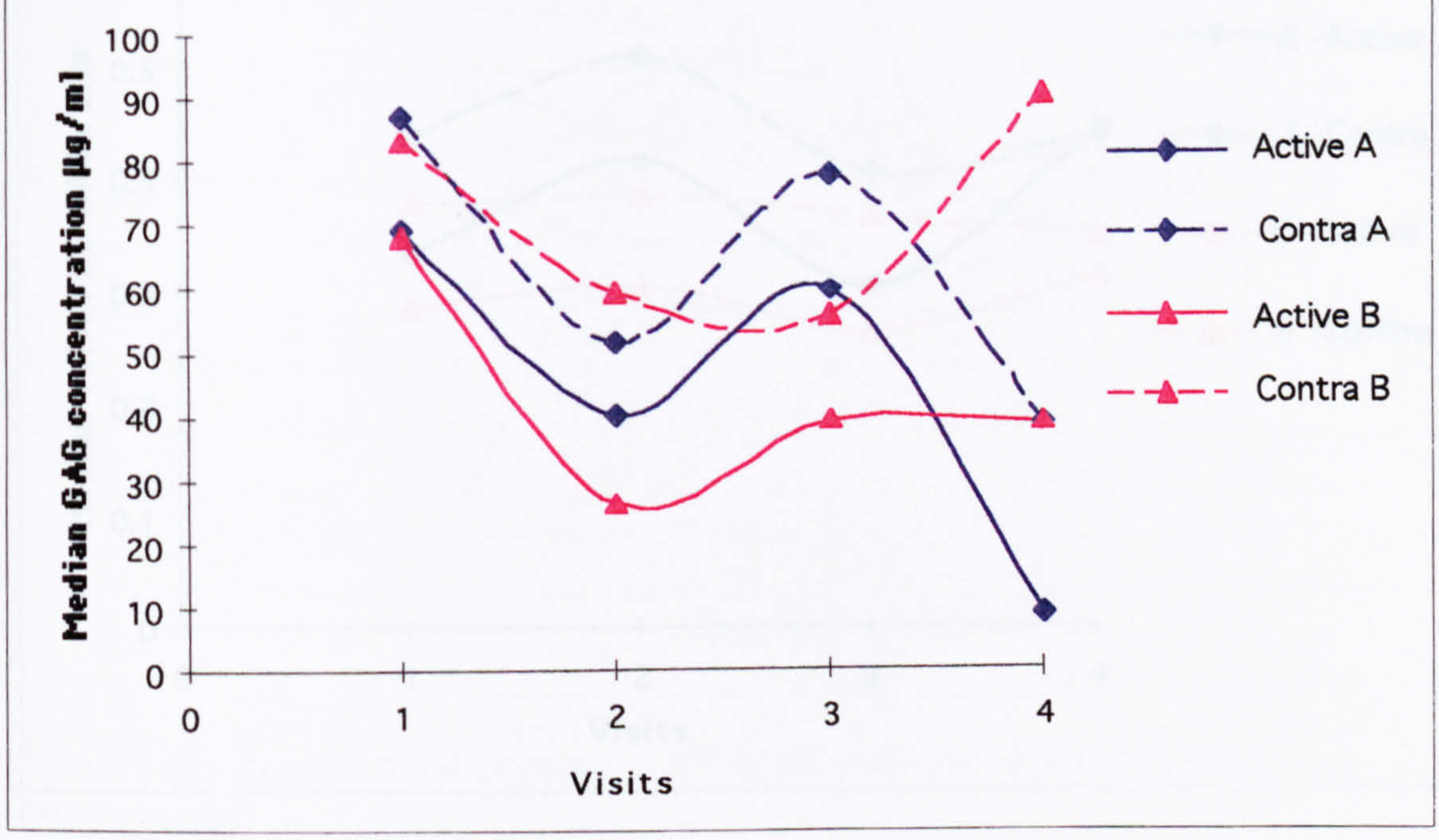

Figure 9-4: Longitudinal changes in median GAG values for matched pairs

e) KS:GAG ratio

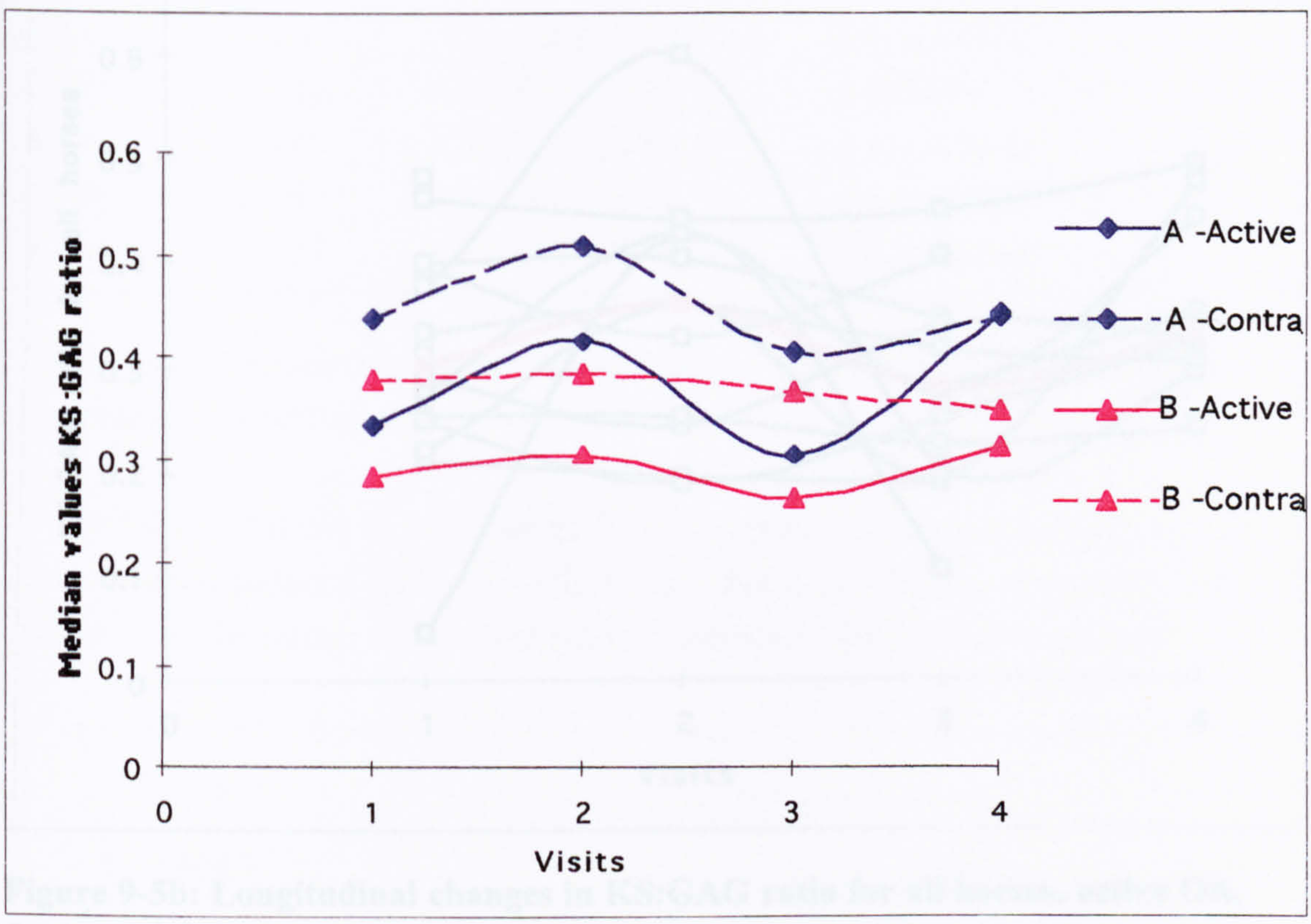

Figure 9-5a :Longitudinal changes in median KS:GAG ratio in matched joints

There were no significant differences between KS, GAG or KS:GAG ratio between the high and low dose treatment groups, at baseline, or at any other sampling time during the trial.

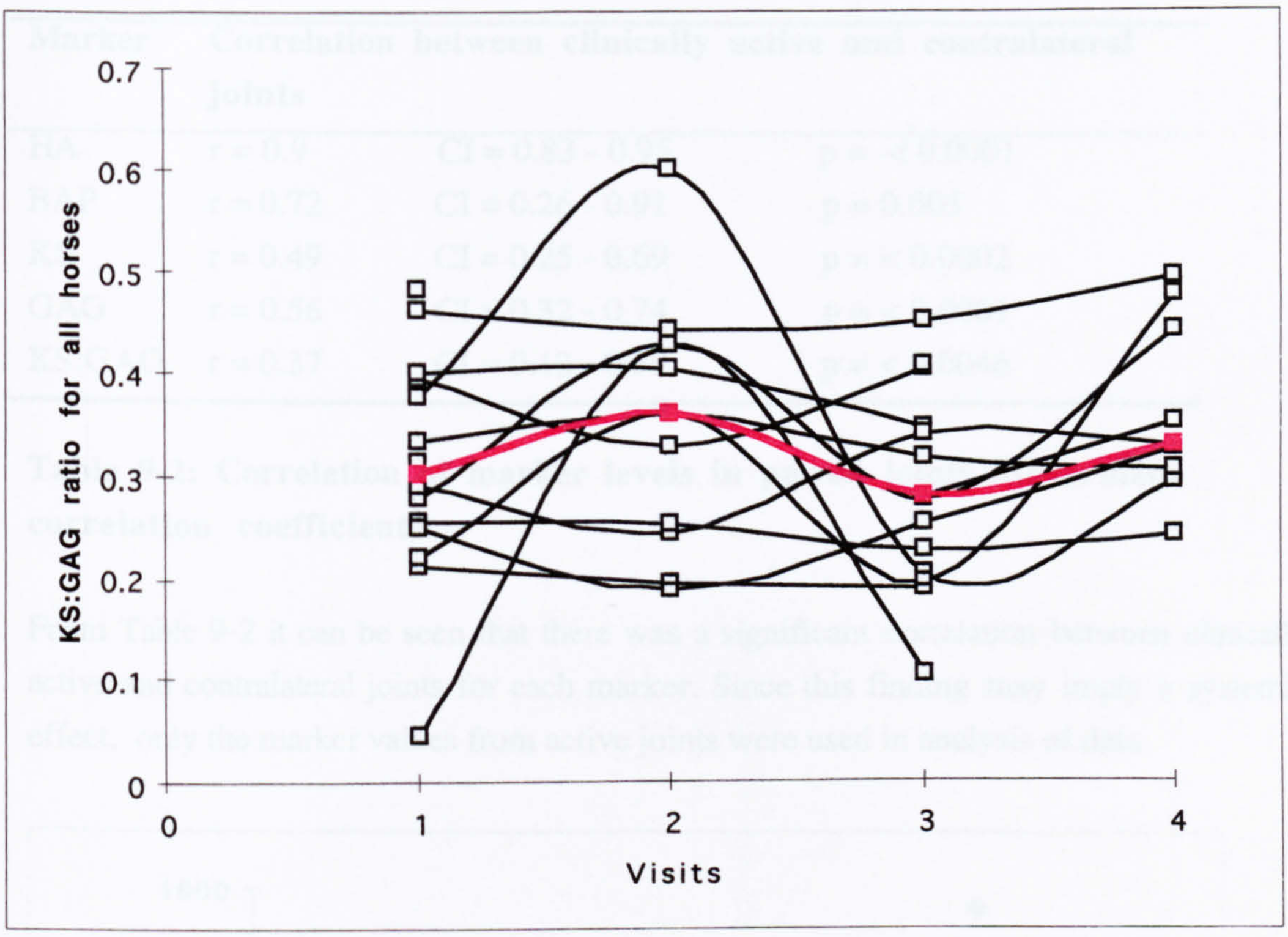

Figure 9-5b: Longitudinal changes in KS:GAG ratio for all horses, active OA, during trial.

Median value = red line.

There was no significant difference in KS:GAG ratio for any horse during the trial.

Marker	Correlation between clinically active and contralateral joints		
HA	r = 0.9	CI = 0.83 - 0.95	p = < 0.0001
BAP	r = 0.72	CI = 0.26 - 0.91	p = 0.005
KS	r = 0.49	CI = 0.25 - 0.69	p = < 0.0002
GAG	r = 0.56	CI = 0.32 - 0.74	p = < 0.0001
KS:GAG	r = 0.37	CI = 0.12 - 0.59	p = < 0.0046

Table 9-2: Correlation of marker levels in paired joints (Spearmans correlation coefficient).

From Table 9-2 it can be seen that there was a significant correlation between clinically active and contralateral joints for each marker. Since this finding may imply a systemic effect, only the marker values from active joints were used in analysis of data.

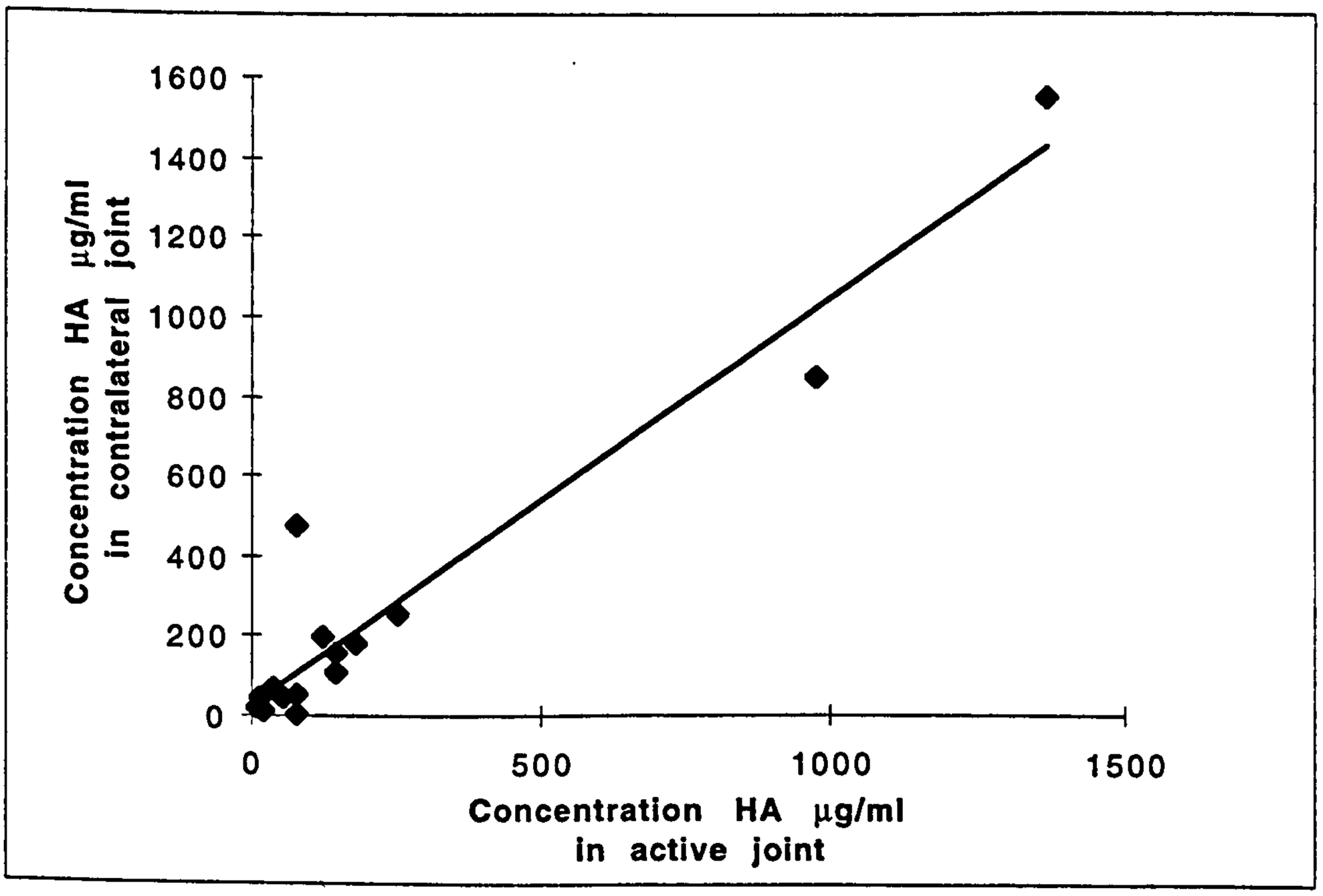

Figure 9-6: Correlation between HASF in clinically active and contralateral joints in CaPPS study

Marker	AI	Change in AI	Scan score	Change in Scan score	Radiography score	Change in rad score	Global score of lameness improvement
HA	NS	NS	**r_s = -0.53** **CI = -0.83, 0.02** **p = 0.05**	NS	**r_s = -0.57** **CI = -0.85, -0.04** **p = 0.032**	NS	NS
BAP	NS	NS	NS	NS	NS	NS	NS
KS	NS	NS	NS	NS	NS	NS	NS
GAG	NS	NS	NS	NS	NS	NS	NS
KS:GAG	NS	NS	NS	NS	NS	NS	NS

Table 9-3: Correlations (Spearmans correlation coefficient) between baseline marker values and other measures of outcome

NS = no significant correlation

Change in marker	Change in AI	Change in Scan score	Change in rad score	Global score of lameness improvement
HA	**r = - 0.76** **CI = -0.93 , -0.35** **p = 0.002**	NS	NS	NS
BAP	NS	NS	NS	NS
KS	NS	NS	NS	NS
GAG	NS	**r = 0.57** **CI = 0.05 , 0.85** **p = 0.031**	**r = -0.56** **CI = -0.84 , -0.02** **p = 0.038**	NS
KS:GAG	NS	NS	NS	NS

Table 9-4: Correlations (Spearmans correlation coefficient) between change in marker values and other measures of outcome

Correlations between SF markers and clinical outcome, radiography and scintigraphy

1. Clinical outcome

There were no significant correlations between any SF markers and global score of lameness improvement.

2. Radiography and scintigraphy

Baseline HASF was significantly negatively correlated with both the scintigraphy global scan score and the radiography global score (Table 9-3).

Increase in concentration of HA during the trial was also significantly correlated with decrease in activity index of the scintigraphic scans. Increase in the concentration of GAG in the SF during the trial was found to be correlated with increase in scintigraphic scan score but with decrease in the radiographic global score (Table 9-4).

Serum marker studies

Serum samples were available for analysis from all horses in the trial i.e. n = 9 in the low dose and n = 10 in the high dose treatment groups.

a) Hyaluronan

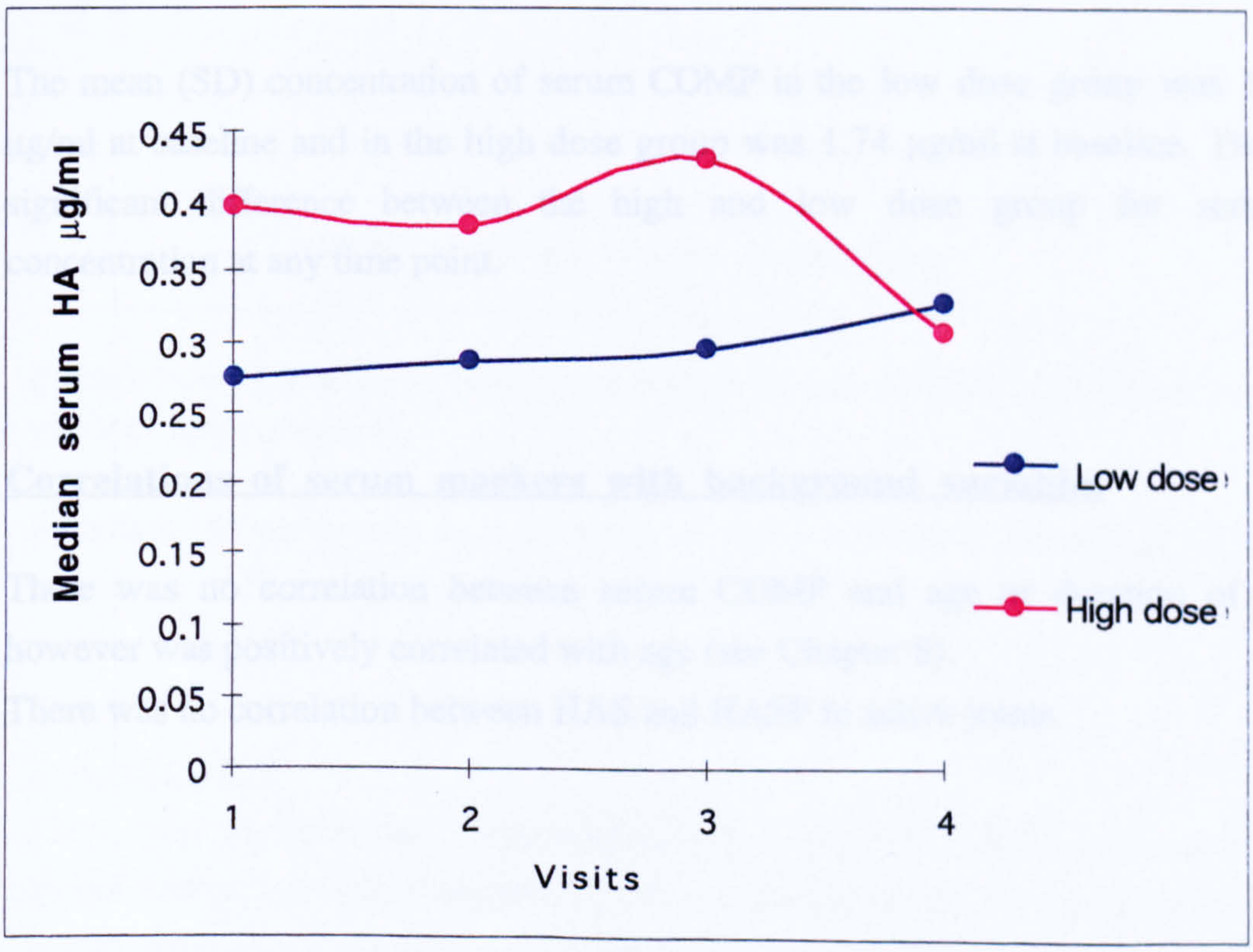

Figure 9-7: Longitudinal change in median HAS for high and low dose groups

There was no significant difference in HAS between groups at any time point (Wilcoxon signed rank).

b) Cartilage oligomeric matrix protein

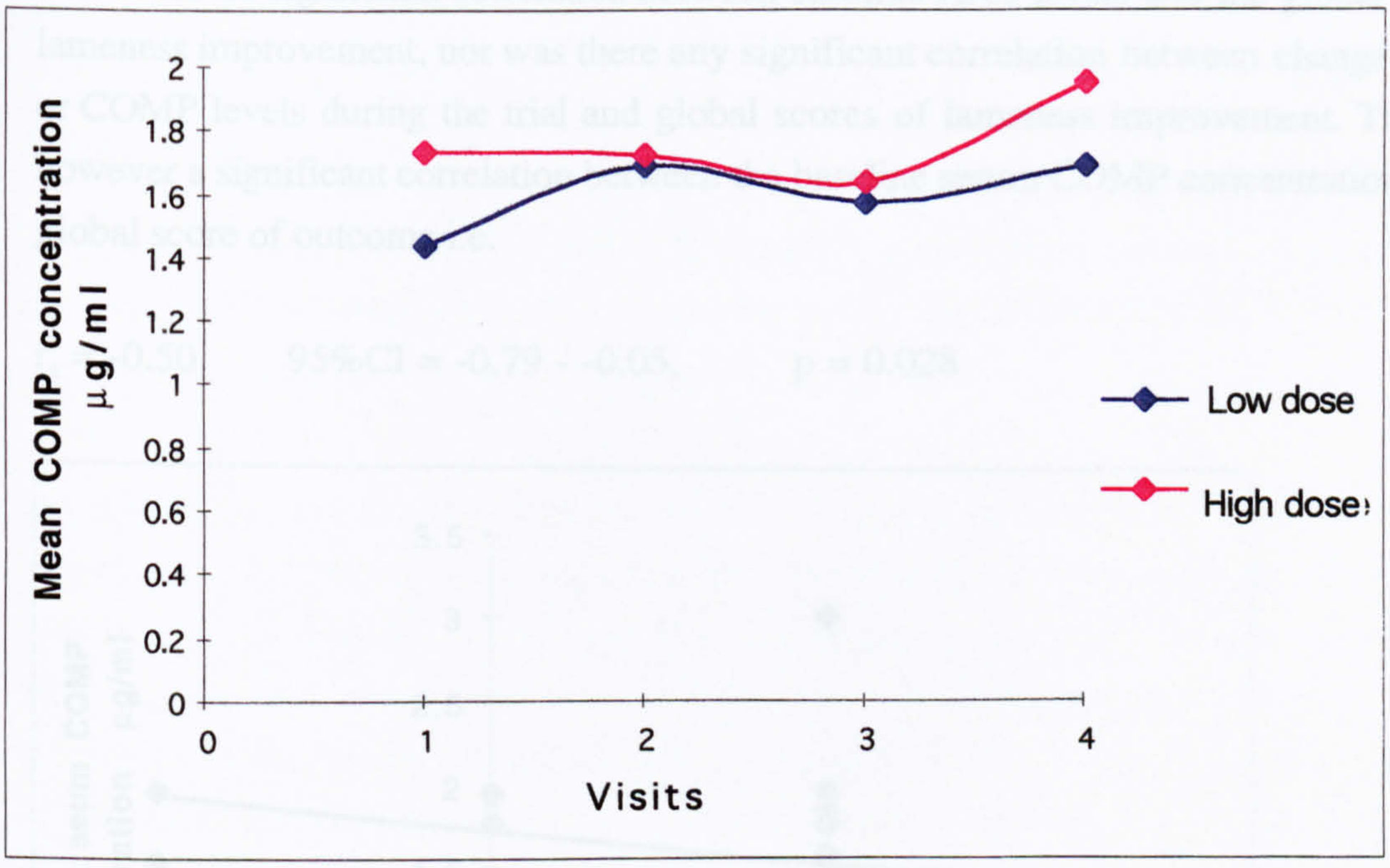

Figure 9-8: Longitudinal mean values of COMP

The mean (SD) concentration of serum COMP in the low dose group was 1.47 (0.39) µg/ml at baseline and in the high dose group was 1.74 µg/ml at baseline. There was no significant difference between the high and low dose group for serum COMP concentration at any time point.

Correlations of serum markers with background variables

There was no correlation between serum COMP and age or duration of OA. HAS however was positively correlated with age (see Chapter 8).

There was no correlation between HAS and HASF in active joints.

Correlation serum marker data with clinical outcome, radiography and scintigraphy

1. Clinical outcome.

There was no significant correlation between baseline HAS levels and the global score of lameness improvement, nor was there any significant correlation between change in HAS or COMP levels during the trial and global scores of lameness improvement. There was however a significant correlation between the baseline serum COMP concentration and the global score of outcome i.e.

r_s = -0.50 95%CI = -0.79 - -0.05, p = 0.028

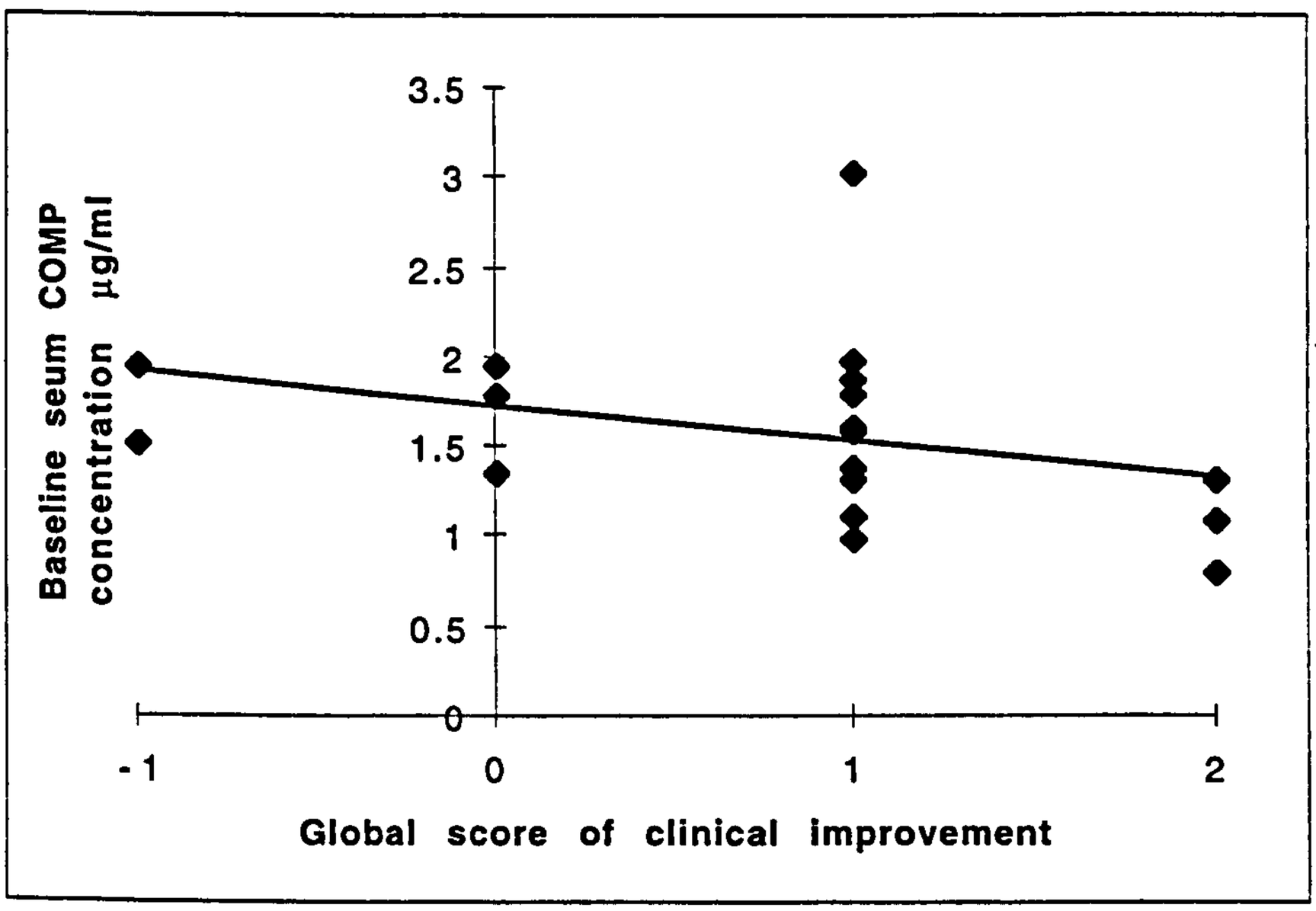

Figure 9-9: Correlation between baseline serum COMP and global score of clinical improvement.

Although the correlation is not strong, it could indicate that baseline serum COMP is predictive of clinical progression in OA.

2. Radiography

There was no significant correlation between HAS or COMP levels and radiographic global scores, nor was there any significant correlation between change in these marker levels with change in radiography scores.

3. Scintigraphy

There was no significant correlation between HAS or serum COMP, either at baseline or in change during the trial, and scintigraphic global scores or scintimetry activity indices.

Discussion

No synovial fluid marker was found to correlate with clinical outcome which was the chosen "gold" standard measure of outcome in the trial although HASF was found to correlate with radiography and scintigraphy.

No differences in outcome between the two treatment groups in the CaPPS trial were identified by the use of biochemical markers in this study. Although this may have been a true result, the potential for a Type II error, largely because of the low power of the study, has been discussed previously (Chapter 4). However there were also no significant temporal changes in any marker for all horses during the trial, indicating that in the stages of OA studied in this trial, none of the markers measured were able to identify any change in OA status.

It was interesting to note that in this cohort of horses, unlike those in the arthroscopy group (Chapter 8) there were significant correlations between marker concentrations in the clinically active OA and the contralateral joints. This may have been because in many cases OA changes occurred bilaterally. It has been previously reported (Dahlberg *et al.* 1994) that this finding may be because of increased weightbearing in the sound limb. It has also been suggested (Belcher *et al.* 1997) that this is because of a systemic effect.
An important consequence of this is that caution should be employed when using a contralateral joint as a normal control .

HASF at entry to the trial was found to correlate, although weakly, with both the scintigraphy and the radiography global score. With the understanding that HASF is reduced in the OA joint compared to normal controls (Chapter 8), it is logical that a decrease in HASF concentration should indicate an increase in the global severity imaging scores. Change in the HASF during the trial correlated more significantly, and negatively, with the change in the scintimetry activity index. The correlation between an increase in activity in the subchondral bone and a decrease in HASF is an indication of a link between bone changes and turnover of HA within the joint.

No other SF markers measured at baseline were predictive of change as measured by other methods. Since the change in scintigraphic scan and radiographic scores were so small in these cases (Chapter 6), this is not surprising. The change in BAP and KS did not show any correlations with other measures but change in GAG concentration was positively correlated to scintigraphic but negatively correlated to radiographic scores. Correlation between serum GAG and scintimetry activity indices has previously been reported (Innes 1997) providing further evidence for a link between bone and cartilage turnover. However since scintigraphic and radiographic scores have been found to be correlated to each other (Chapter 6) it is difficult to understand why the correlation found here between GAG and radiographic score should be negative. In Chapter 8 cross-sectional differences between OA and normal joints for GAG were not significant and in Chapter 6 change in radiography score during the trial period was minimal. In view of these findings, interpretation of these correlations has to remain inconclusive.

No SF marker was found to correlate with clinical outcome which was the chosen "gold" standard measure of outcome in the CaPPS trial. However, all the horses in the trial had OA of more than 3 months duration and it may be that in this chronic condition, none of the markers measured here could demonstrate significant change.

HAS did not correlate with any other outcome measure, unlike in man where it has been found to be predictive of OA progression. Single sample measurement of this marker does not appear to be useful in the horse, since it has a wide normal variation and change throughout the day. It is also important to note that the serum levels of HA did not correlate with those in the SF of clinically active OA joints. However, serum COMP was found to correlate with the global score of lameness improvement indicating that a high serum COMP at baseline is predictive of clinical deterioration.

In this study, none of the markers measured has been useful in the identification of longitudinal change in OA. HASF has shown interesting correlations with scintigraphy and radiography, and a single baseline measurement of serum COMP may be said to be predictive of clinical change in OA. This study has been limited by small numbers, and these preliminary results would merit further investigation in a larger trial.

Summary

1. No significant longitudinal changes were discovered in any marker measured during the 9 months of the trial.

2. Single measurements of HASF correlated with radiography and scintigraphy scores and increase in HASF correlated with decrease in the scintimetry activity indices. Single baseline serum COMP measurement was predictive of clinical change.

3. No difference in outcome between high and low dose treatment groups in the CaPPS trial was identified by the use of biochemical markers.

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Chapter Ten

Are all joints the same?

Variations between normal equine joints

Introduction

The purpose of this study was to investigate whether metabolic differences exist between normal equine joints. OA in the horse has traditionally been classified according to the type of disease occurring in joints of high or low range of motion (See Chapter 1 Table 1-1). During the assessment of the CaPPS trial it seemed that (Chapter 5 Figure 5-6 and 5-7) horses with OA in low motion joints (TMT, PIP, and DIP) were more likely to show clinical improvement than those with OA affecting high motion joints (MCP and MC) but the clinical data was too small to determine this accurately. Variations have been found in marker levels (Chapter 7) and ratios of glycosaminoglycans in the synovial fluid of normal equine metacarpophalangeal (MCP), proximal interphalangeal (PIP), and distal interphalangeal (DIP) joints (Fuller *et al.* 1996) but the reason for this difference is not known. It may relate to a difference in metabolism between the cartilage in these joints. This may provide evidence that cartilage physiology is specific to the function required by the particular joint. In human research, studies indicate that osteoarthritis (OA) of different joint sites may involve a different set of risk factors, which raise important issues about aetiopathogenesis (Kirwan *et al.* 1994). It seems that the localisation of OA within and between joints may be controlled by a balance of systemic and local factors, and therefore OA of one joint may have a different aetiology to that of another. It may also follow that efficient therapy in OA would differ according to the joint affected.

In many clinical trials performed in horses, cases of OA affecting a variety of different joints are included, and responses to treatment of these joints directly compared. The existence of joint differences would add another source of variability to trials thereby decreasing the accuracy of the assessment. Therefore, in this chapter, findings from studies investigating differences occurring between normal equine joints will be reported.

Variations in metabolism

Differences in the metabolism of various joints were explored by investigating the response of cartilage from different equine joints to the same challenge by a cytokine. Interleukin - 1 (IL-1) is a protein secreted by the cells of the monocyte-macrophage line, although it has been found to be produced in smaller quantities by the chondrocytes themselves. It has been found to stimulate the proteolytic pathways of extracellular matrix breakdown while subduing the synthesis leading to new matrix formation (Pelletier *et al.* 1993). It stimulates the expression of proteases including stromelysin, collagenases, and plasminogen activating factor. This has been confirmed *in vivo* by intra-articular injection of the cytokine (Lotz *et al.* 1995). This cytokine was chosen for use in this study since it has been found to occur in equine synovial fluid at a higher concentration in osteoarthritic (Alwan *et al.* 1991) than in normal joints and it is thought therefore to be involved in the pathogenesis of the disease. Also, recently it has been shown in man that the joint and joint site from which cartilage is sampled may affect responses to IL-1 (Hauselmann *et al.* 1996; Hauselmann *et al.* 1993). A dose rate of 10ng/ml was used since this has been shown in previous studies to elicit a response in cartilage from horses in the same age group i.e. mean age 12 years (MacDonald *et al.* 1992). In this study the recombinant human IL-1α was used; however previous studies have shown that there is a considerable degree of homology between human and equine IL-1α (Morris *et al.* 1990).

Concentration of total sulphated glycosaminoglycans (GAG) in the supernatant can be measured to quantify the degree of degradation occurring in the cartilage explants as a result of the catabolic cytokine. During cartilage breakdown, as described in detail in Chapter 1, it is the upregulation of metalloproteinases by these cytokines, and then the subsequent activation of these enzymes by serine proteases that lead to the release of glycosaminoglycans from the cartilage matrix. One of the metalloproteinases involved in this process is MMP-2 or the 72kDa gelatinase. This is produced by the chondrocytes and once activated, degrades Type IV and denatured Type II collagen. The measurement of metalloproteinase activity in cartilage explants will elucidate whether any potential differences in cartilage response are related to differences in enzyme production.

Cartilage explant cultures have been found to be a good model for the analysis of matrix degeneration (Tyler *et al.* 1990). Cartilage culture has several advantages over that of isolated chondrocytes including the fact that the extracellular matrix is similar to that *in vivo*, and the chondrocytes are not exposed to exogenous proteolytic activity.

Structural variations

Studies arising from the work on normal joint differences included those to determine differences in other parameters in normal MCP PIP and DIP joints which may have an effect on SF marker levels and cartilage responses, i.e. cartilage thickness and mass.

Aims

1. To investigate the hypothesis that cartilage from different normal equine joints responds differently to the same catabolic challenge. This investigation was carried out using *in vitro* cartilage explant culture techniques. Release of GAGs from the explants and activity of the metalloproteinase MMP-2 were measured to demonstrate the effects of IL-1α stimulation.

2. To establish whether basic structural differences occurring in joints may be related to marker variations previously reported.

Materials

48 well tissue culture plates	Life Technology Ltd., Paisley, UK.
Dulbecco's modified Eagle's Medium	Life Technology Ltd., Paisley, UK.
Human recombinant IL-1α	Sigma Chemical Co., Poole Dorset, UK.
Streptomycin	Sigma Chemical Co.
96 well polystyrene assay plate	Nunc (ImmunoPlate Maxisorp), Life Technology Ltd., Paisley, UK.
Dimethylmethylene blue dye	Serva, Heidelberg, FRG
Papain	Sigma Chemical Co.
Na2HPO4	Sigma Chemical Co.
Bovine tracheal chondroitin sulphate A	Sigma Chemical Co.
NaCl	Sigma Chemical Co.
Glycine	Sigma Chemical Co.
Automated plate reader	Labsystems Multiskan, Life Sciences International.

Methods

Variations in metabolism :

Horse	Type	Approximate age (years)
1	Pony	10
2	Pony	10
3	ThoroughbredX	15
4	Pony	20
5	ThoroughbredX	10
6	ThoroughbredX	10

Table 10-1: Distribution of types and ages of horses from which cartilage explants were harvested.

Tissue culture

Full thickness cartilage samples from normal equine joints were taken from both articular surfaces of each of the metacarpophalangeal (MCP), proximal interphalangeal (PIP), and distal interphalangeal (DIP) joints from six horses destroyed for reasons unrelated to locomotor system disorders (Table 10-1). The mean age of the horses was 12 years (range 10-15). The samples were harvested using aseptic techniques immediately following humane destruction. These were maintained in Dulbecco's modified Eagles medium (DMEM) to which antibiotic agents had been added. Under sterile conditions the cartilage samples were diced into uniform 1mm explants and randomised before placing in duplicate into a 36 well flat bottomed culture plate. 1ml of DMEM was added to each well and the explants were then cultured in a humidified incubator at 37°C, in an atmosphere containing 5% CO_2 . After 24 hours the explant supernatant samples were removed and stored at -20°C. The medium was replaced in the control samples by fresh DMEM , and in each paired stimulated sample by DMEM to which 10ng/ml recombinant human IL-1 (Sigma) had been added. This procedure was repeated at the end of day 2 and 3. After this the medium was replaced daily in all samples by fresh DMEM only. At the end of this seven day period the remaining explants were also removed from the culture plate and stored at -20°C until ready for assay.

Glycosaminoglycan assay

The remaining cartilage explants were first digested overnight at 65°C with a papain buffer consisting of 2% papain (Sigma), 0.5M Cysteine-HCl, 0.05M EDTA, and 0.4M Na acetate. The supernatants and the digested cartilage explants were then analysed for total glycosaminoglycans (GAG) by a direct colorimetric method (Farndale *et al.* 1986).

Gelatin zymography

Samples of the supernatants were sent to Dr P. Clegg (Liverpool University) where gelatin zymography was performed to determine gelatinase activity as described in Clegg *et al* (Clegg *et al.* 1997) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a vertical gel apparatus (Bio-Rad, Hemel Hempstead, UK) according to the method of Laemmli (1970), with the modification that gelatin was included in the resolving gel (Hibbs et al. 1985). Gelatin was co-polymerised into 0.75 mm thick, 7.5% polyacrylamide gels at a final concentration of 2.5mg/ml. Cell culture supernatants samples were diluted 4:1 in a Tris/HCl sample buffer containing 1.5% SDS, 5% glycerol and 0.005% Bromophenol blue, pH 6.8 and incubated at 37°C for one hour prior to electrophoresis. Each dilution (7.5=B5l) was loaded in a well and samples electrophoresed at 200V for approximately 45 minutes. The gels were washed for one hour at room temperature in 2.5% Triton X-100, rinsed in distilled water and incubated for 18 hours at 37°C in a reactivation buffer containing 50mMTris/HCl, 50m= M CaCl2, 10mM NaCl, 0.05% Brij35, pH7.6. Following incubation, the gels were stained with 2% Coomassie brilliant blue R-200 followed by destaining in a mixture of 7% (v/v) glacial acetic acid, 30% (v/v) methanol and 63% (v/v) distilled water. The gels were dried in a cellophane sandwich. Molecular weight standard markers (Mark 12 wide range protein standards: Novex) were run on each gel which were stained prior to the gel being washed in Triton.

Image analysis of gelatin zymograms

Computer assisted image analysis of the zymography gels was performed as described in Clegg et al (Clegg *et al.* 1997). For each gel, the activity value (AV) of each MMP-2 band was determined as the product of its area and mean intensity using an image analysis program (NIH image 1.44). The relative activity value was calculated by division of the AV of the band in question by the AV of the MMP-2 band in the foetal calf serum standard run on each gel.

Statistics

Statistical analysis on the GAG samples was by repeated measures ANOVA. The numbers were too few to test normality but since the statistics were done on rates it could be assumed that these were normally distributed. Mann Whitney nonparametric tests were used to compare population of samples for the MMP-2 results.

Structural variations :

a) Cartilage thickness

Differences in cartilage thickness in the MCP, PIP, and DIP joints of normal horses was investigated. Each of these three joints from the same foreleg of six horses was sectioned in a frontal plane perpendicular to the weight bearing surface of the joint and the thickness of the cartilage of the proximal and distal joint surface was measured at three different points across the joint. In three of these horses the same measurements were also carried out on the same three joints from one hindleg, to ascertain whether any difference exists between fore and hind leg cartilage thickness.

b) Cartilage Mass

The cartilage from each entire joint surface of each of the MCP, PIP, and DIP joints from the same leg from six horses was dissected free with a scalpel, and then weighed.

Statistical analysis was carried out by ANOVA.

Results

Variations in metabolism:

Glycosaminoglycans

Results were expressed as percentage total GAG, i.e. GAG released into the supernatant as a % of the total GAG in the system. (See Tables 10-2 and 10-3).

Horse		1	2	3	4	5	6
MCP	Control	9.75	13.13	9.62	6.13	17.24	14.96
	Stimulated	16.04	22.79	16.80	8.79	21.01	24.56
	Difference	6.29	9.67	7.18	2.66	3.77	9.59
PIP	Control	17.06	20.89	35.61	14.03	18.89	13.48
	Stimulated	21.41	53.09	51.71	20.42	38.60	29.57
	Difference	4.36	32.19	16.10	6.38	19.71	16.09
DIP	Control	21.23	39.59	36.23	21.13	34.31	34.61
	Stimulated	30.97	86.22	46.82	24.07	66.15	58.59
	Difference	9.73	46.64	10.58	2.93	31.84	23.97

Table 10-2: Total % GAG release from control and stimulated cartilage explants, at Day 7, for all horses.

(MCP = metacarpophalangeal joint, PIP = proximal interphalangeal joint

DIP = distal interphalangeal joint)

		Day 1	Day 7
MCP	Control	3.40	11.80
	Stimulated	3.17	18.33
PIP	Control	6.52	19.99
	Stimulated	6.45	35.80
DIP	Control	9.03	31.18
	Stimulated	9.83	52.14

Table 10-3: Mean total % GAG release from control and stimulated cartilage explants at day 1 and day 7.

There was a significant difference between the rate of release of GAG from the unstimulated control samples from the different joints over the 7 day period ($p=0.0004$) with the cartilage from the DIP joint releasing GAG at the higher rate than the PIP which in turn released GAG at a higher rate than the MCP joint (See Figure 10-1).

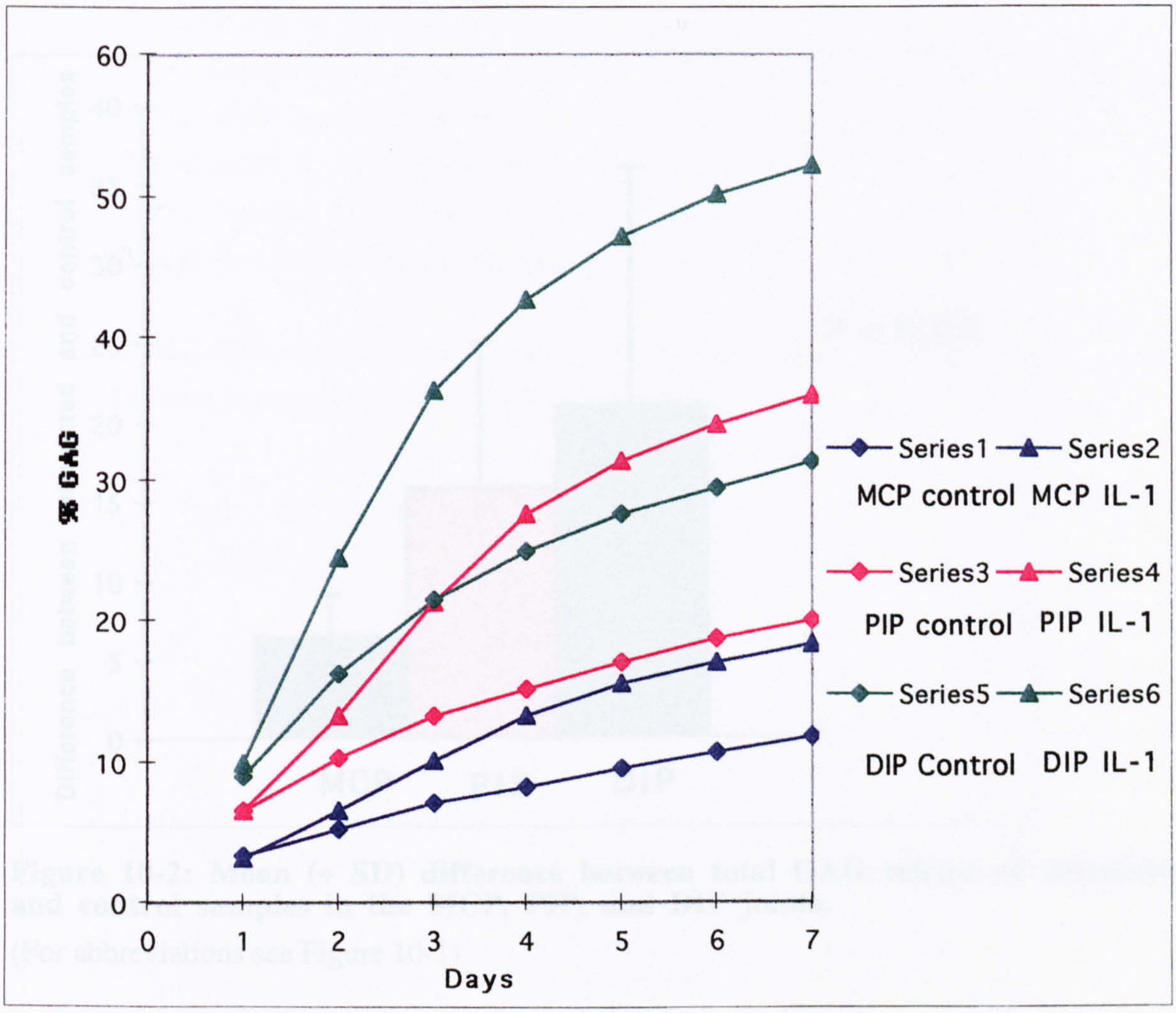

Figure 10-1: Mean cumulative % GAG release from control and stimulated samples from the MCP, PIP, and DIP joints.

(GAG = total glycosaminoglycans, MCP = metacarpophalangeal joint, PIP = proximal interphalangeal joint, DIP = distal interphalangeal joint, IL-1 = interleukin -1)

All the cartilage explants responded to the IL-1 stimulus by releasing GAG at a higher rate than unstimulated explants. One explant sample responded at a lesser rate than the others, this sample was from the oldest horse in the group. The rate of release of GAG in specific response to IL-1 stimulation was calculated as the difference between the rate of release of GAG in the control samples and that in the stimulated ones. There were significant differences between joints in relation to total GAG released, over the whole 7 day period of IL-1 stimulation and then recovery. ($p = 0.02$ ANOVA) (See Figure 10-2.)

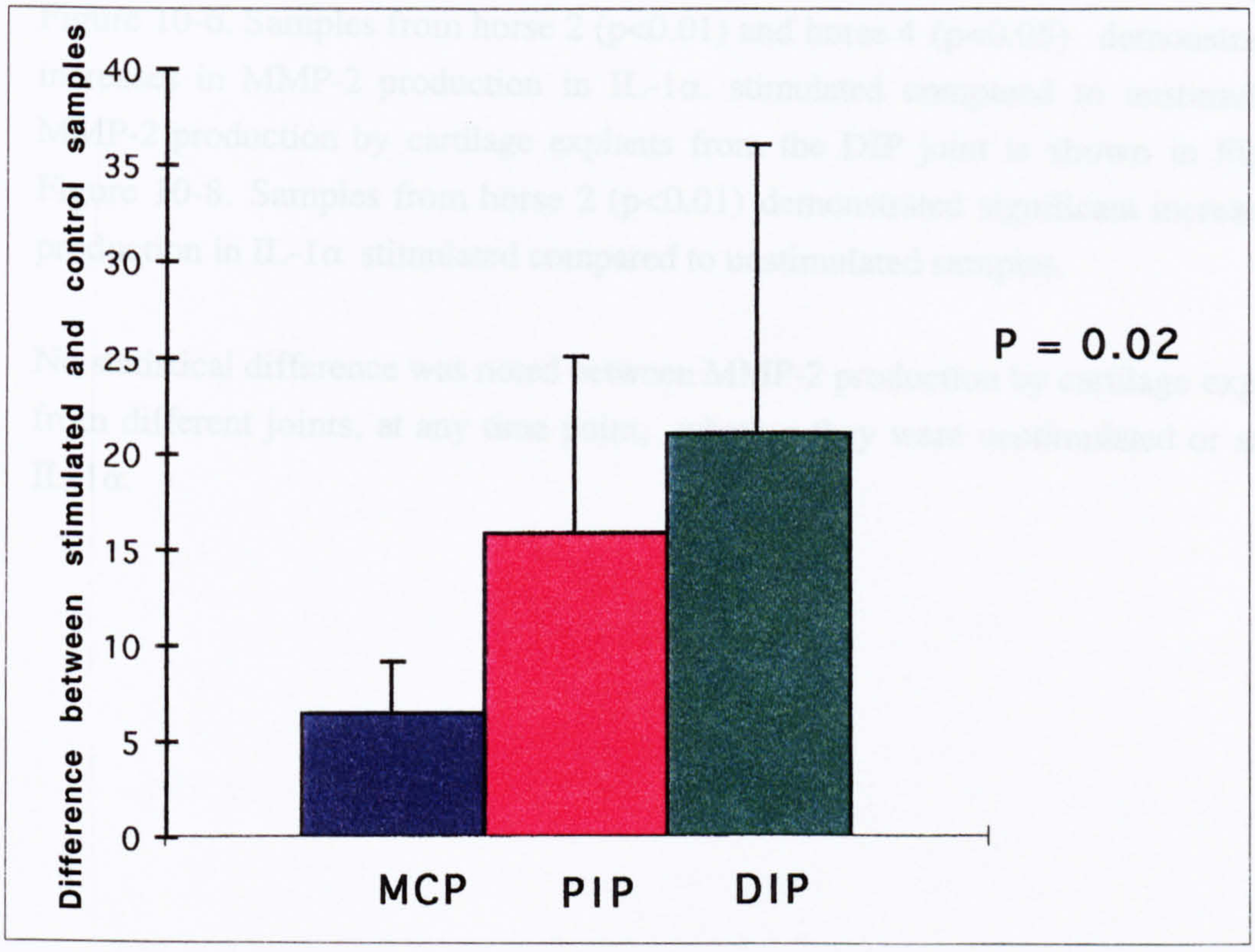

Figure 10-2: Mean (+ SD) difference between total GAG release of stimulated and control samples in the MCP, PIP, and DIP joints.

(For abbreviations see Figure 10-1)

The difference in response between samples in different joints over the 3 day period of stimulation of the cartilage was significant ($p = 0.03$), however the difference in recovery rates after withdrawal of the IL-1 was not significant.

MMP-2

Results for MMP-2 are expressed as a relative activity value (as compared with the foetal calf serum standard). MMP-2 production by cartilage explants from the MCP joint is shown in Figure 10-3 and Figure 10-4. Samples from horses 2 and 5 demonstrated significant increases in MMP-2 production ($p<0.01$) in IL-1α stimulated compared to unstimulated samples.

MMP-2 production by cartilage explants from the PIP joint is shown in Figure 10-5 and Figure 10-6. Samples from horse 2 ($p<0.01$) and horse 4 ($p<0.05$) demonstrated significant increases in MMP-2 production in IL-1α. stimulated compared to unstimulated samples. MMP-2 production by cartilage explants from the DIP joint is shown in Figure 10-7 and Figure 10-8. Samples from horse 2 ($p<0.01$) demonstrated significant increases in MMP-2 production in IL-1α stimulated compared to unstimulated samples.

No statistical difference was noted between MMP-2 production by cartilage explants obtained from different joints, at any time point, whether they were unstimulated or stimulated with IL-1α.

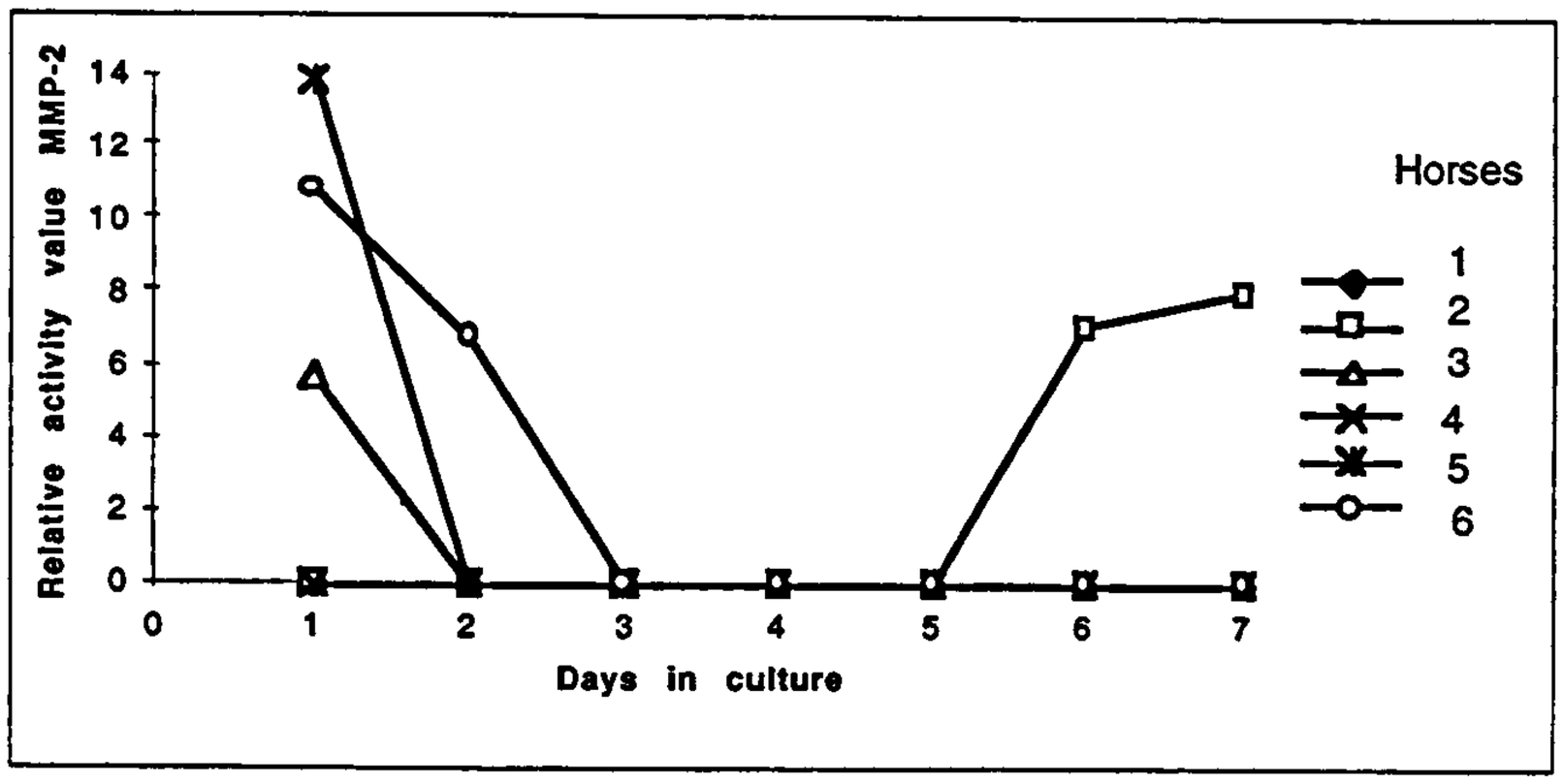

Figure 10-3: Relative activity values of MMP-2 in unstimulated control cartilage explants from MCP joint

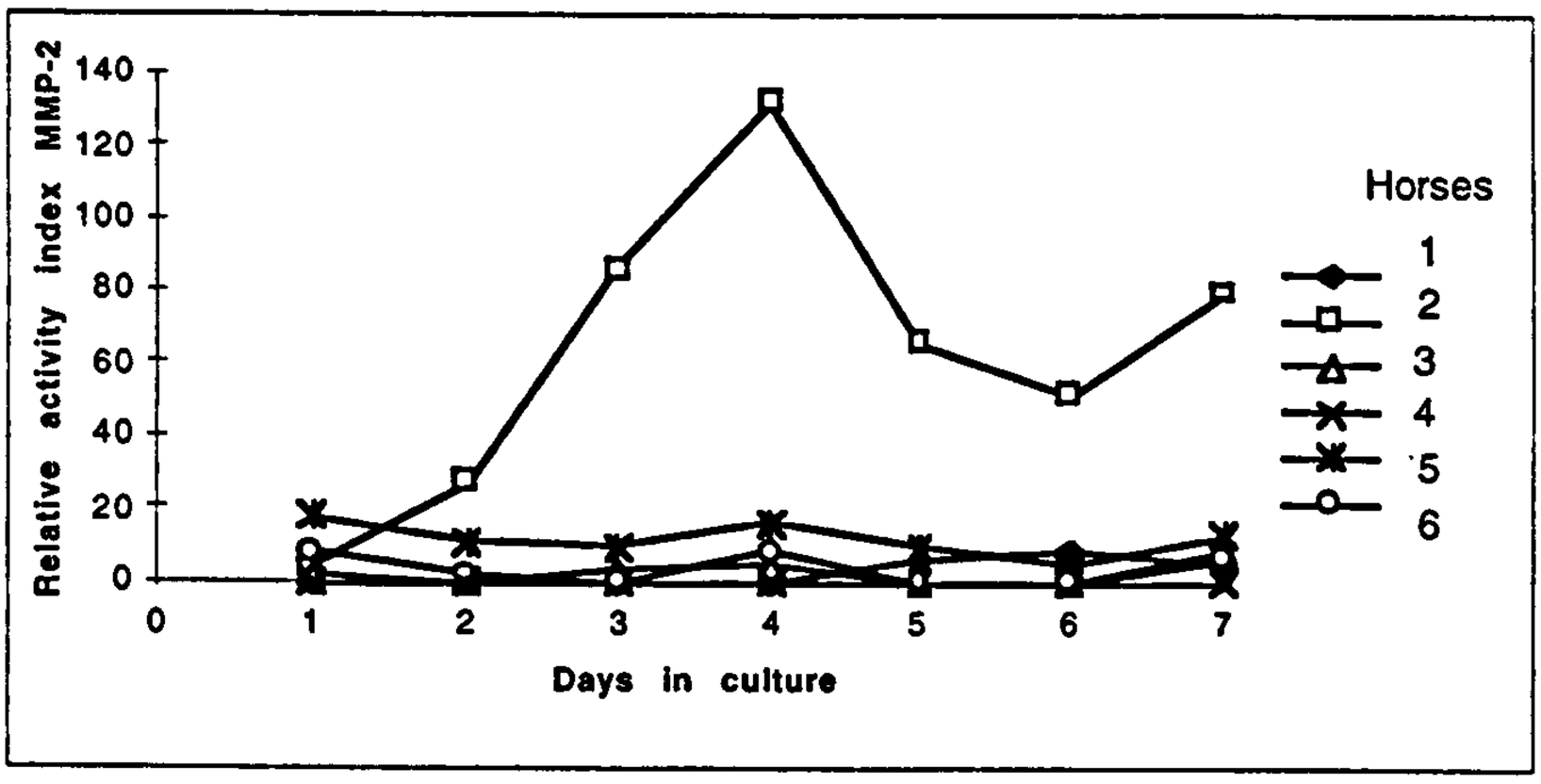

Figure 10-4a: Relative activity values of MMP-2 in all IL-1 stimulated cartilage explants from MCP joint

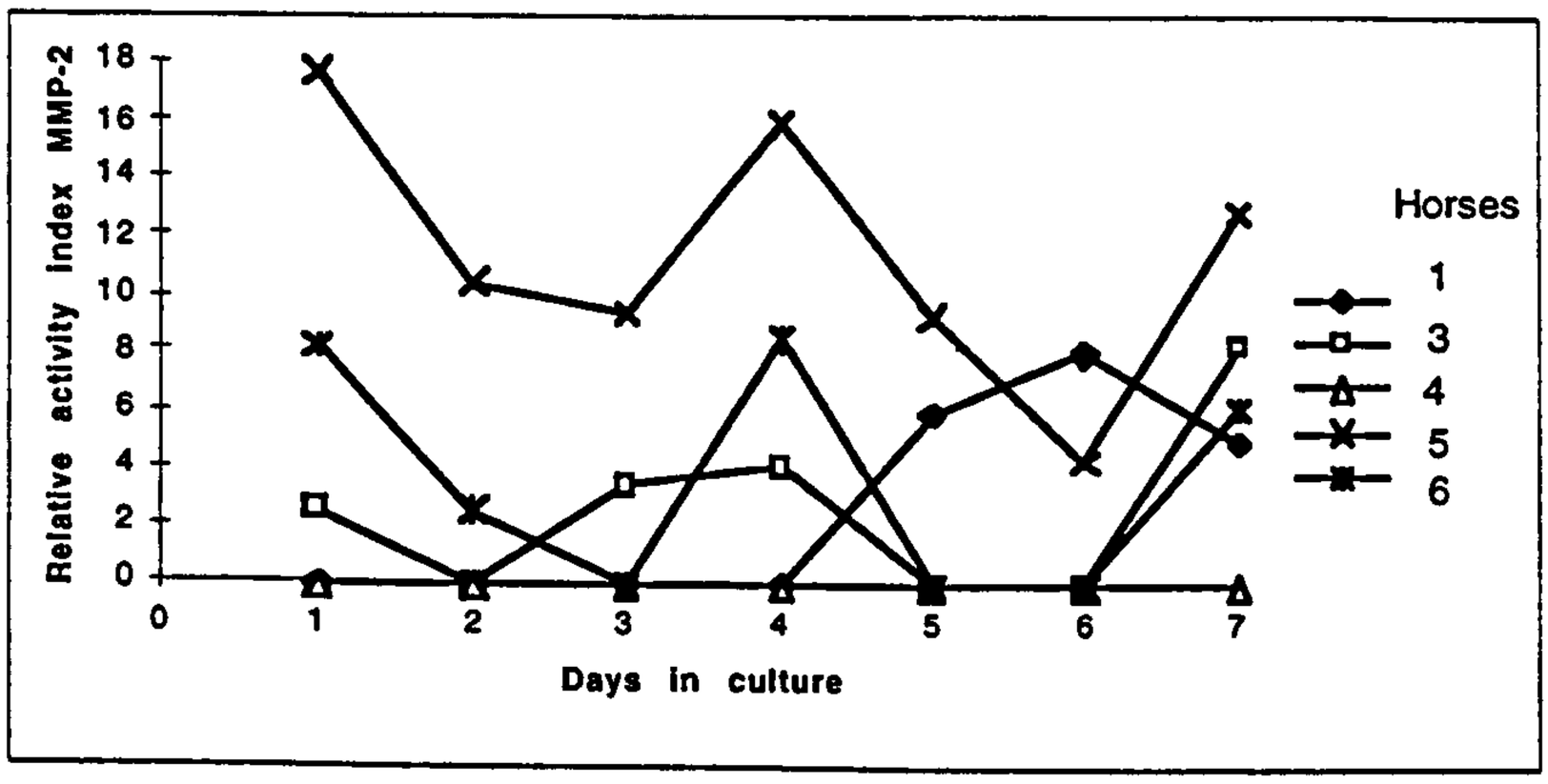

Figure 10-4b: Relative activity values of MMP-2 in IL-1 stimulated cartilage explants from MCP joint excluding those from Horse 2

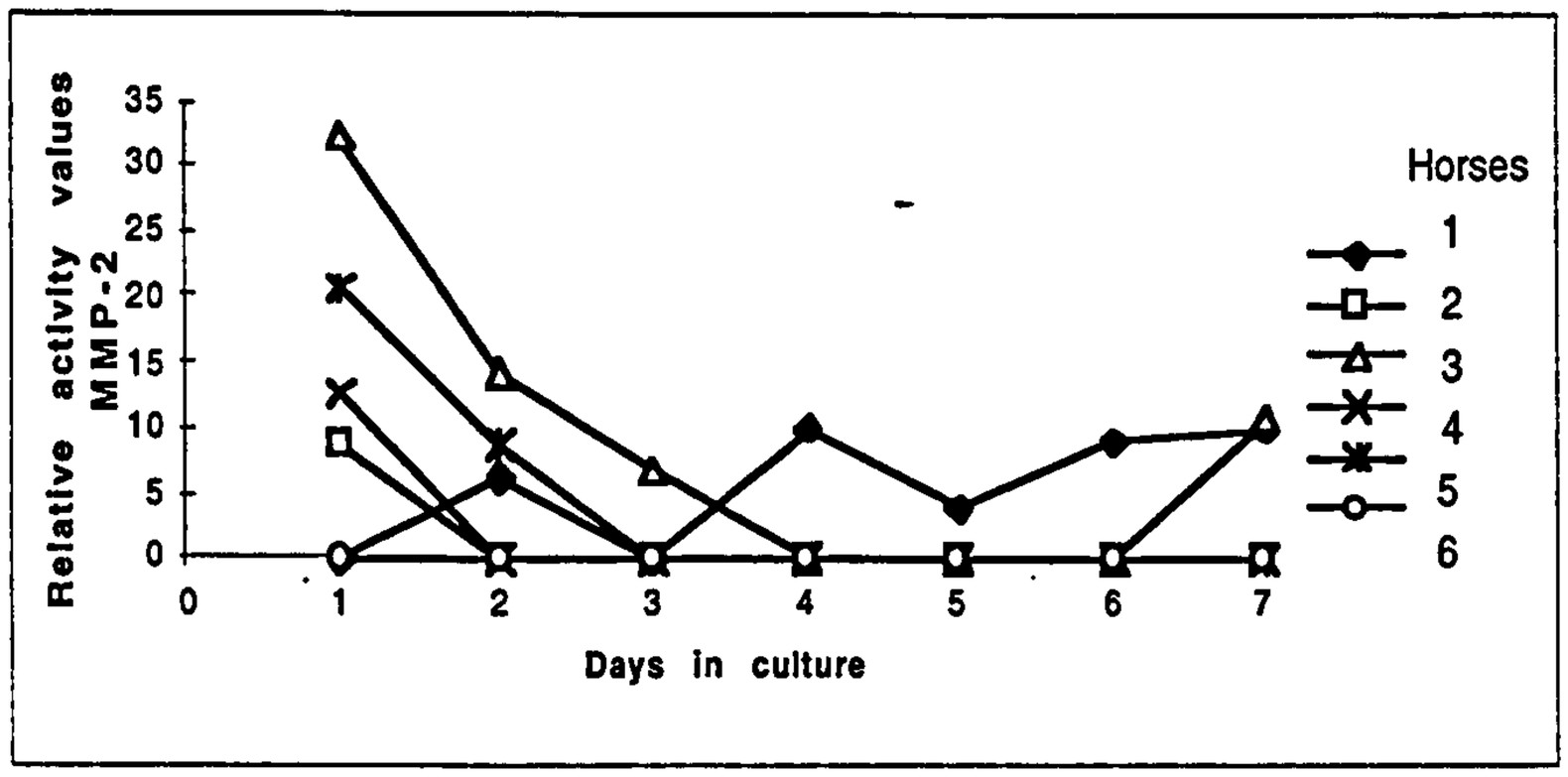

Figure 10-5: Relative activity values of MMP-2 in unstimulated control cartilage explants from PIP joint

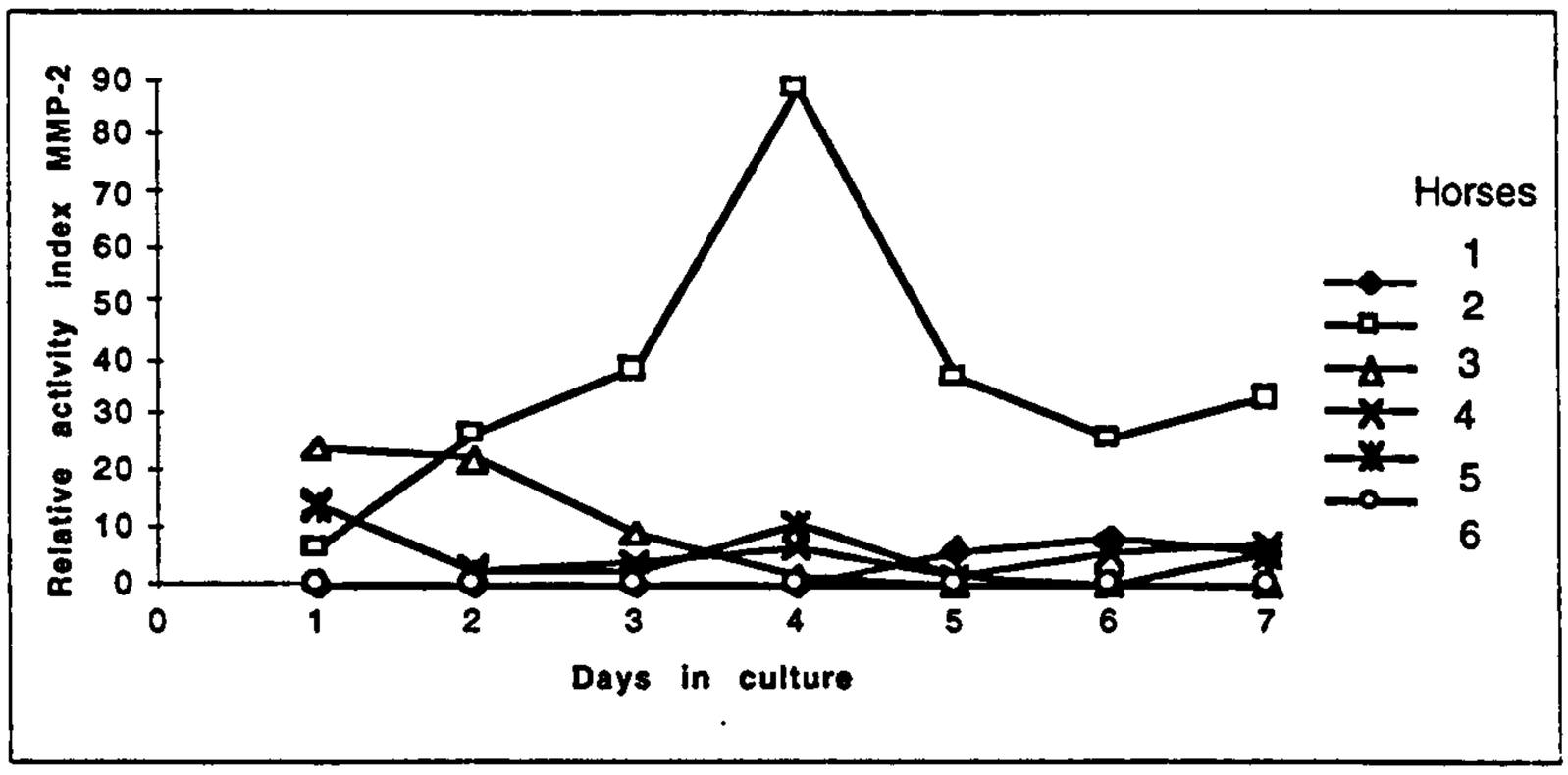

Figure 10-6a: Relative activity values of MMP-2 in all IL-1 stimulated cartilage explants from PIP joint

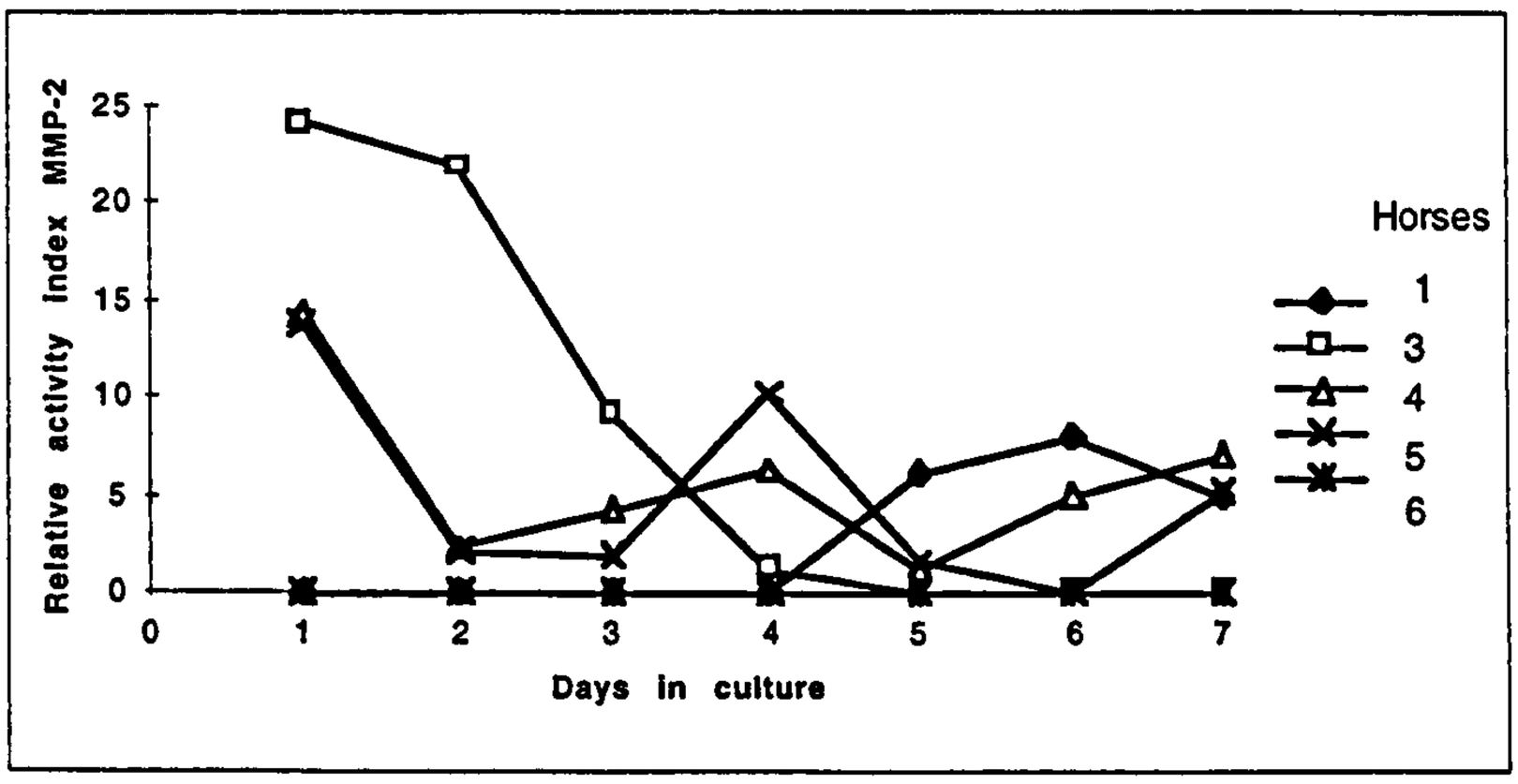

Figure 10-6b: Relative activity values of MMP-2 in IL-1 stimulated cartilage explants from PIP joint excluding those from Horse 2

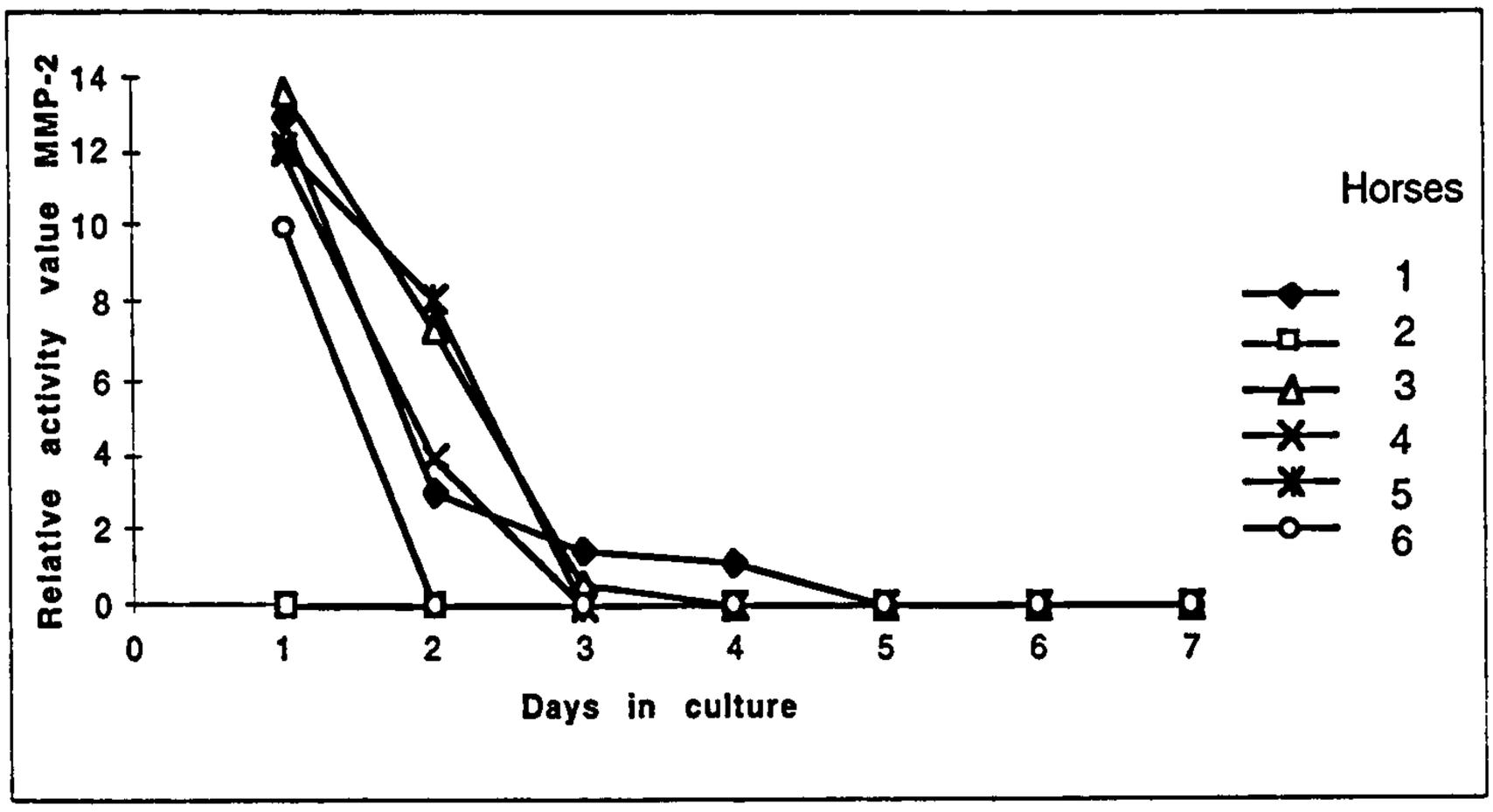

Figure 10-7: Relative activity values of MMP-2 in unstimulated control cartilage explants from DIP joint

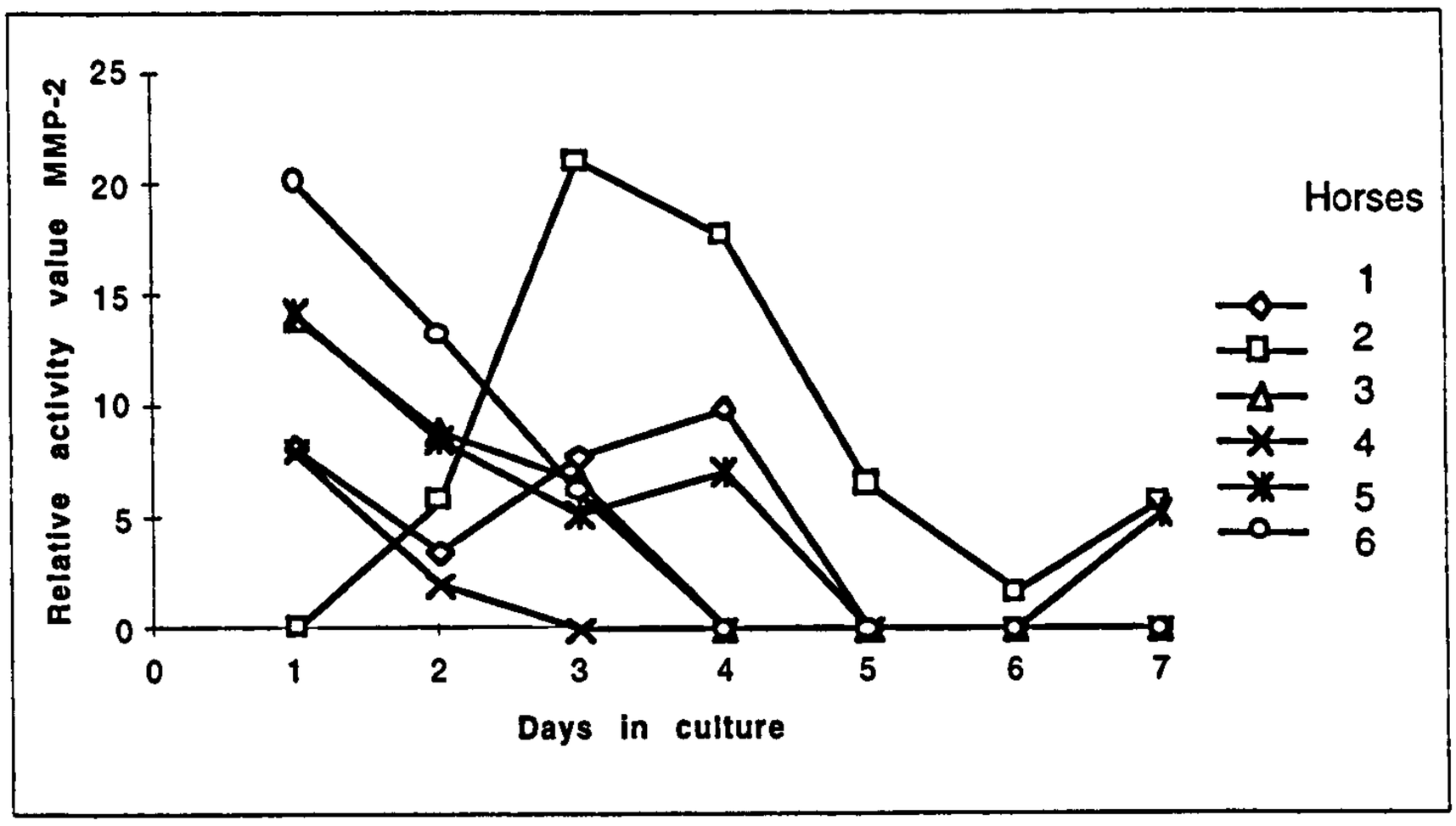

Figure 10-8: Relative activity values of MMP-2 in IL-1 stimulated cartilage explants from DIP joint

Structural variations

The mean (SD) value for cartilage thickness in mm for each joint measured summarised in Table 10-4:

Joint	MCP	PIP(Fore)	DIP(Fore)	MTP	PIP(Hind)	DIP(Hind)
Proximal	**0.93**(0.09)	**1.11**(0.11)	**1.98**(0.19)	**0.88**(0.08)	**1.03**(0.12)	**1.5**(0.87)
Distal	**0.99**(0.07)	**1.24**(0.12)	**2.10**(0.21)	**0.87**(0.11)	**0.99**(0.02)	**1.75**(1.01)

Table 10-4: Mean cartilage thickness (SD) in mm of proximal and distal cartilage surfaces of normal MCP/MTP, PIP, and DIP joints.

MCP = metacarpophalangeal , MTP = metatarsophalangeal,

PIP = proximal interphalangeal, DIP = distal interphalangeal

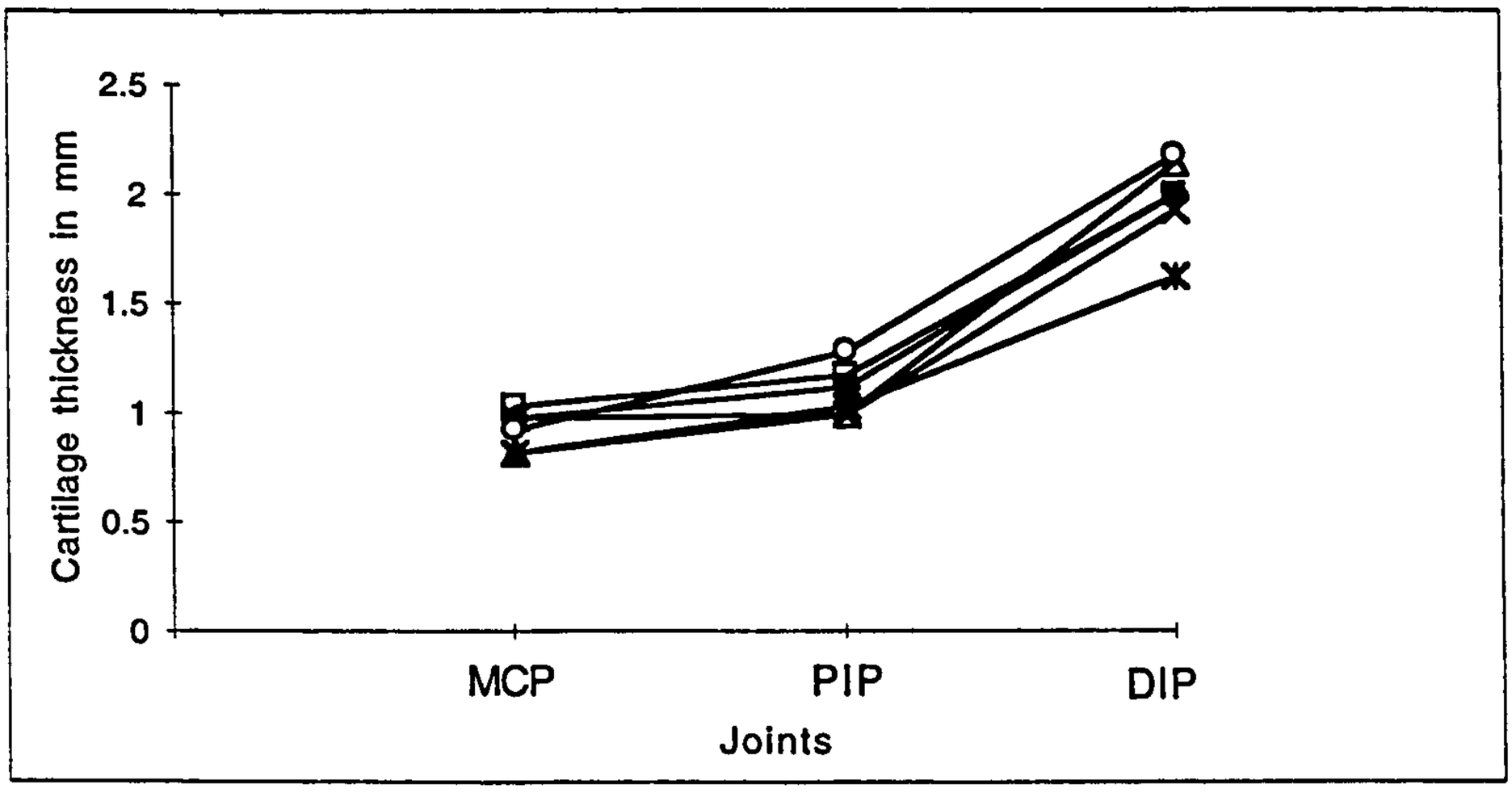

Figure 10-9: Cartilage thickness in proximal surface of matched normal MCP, PIP, and DIP joints

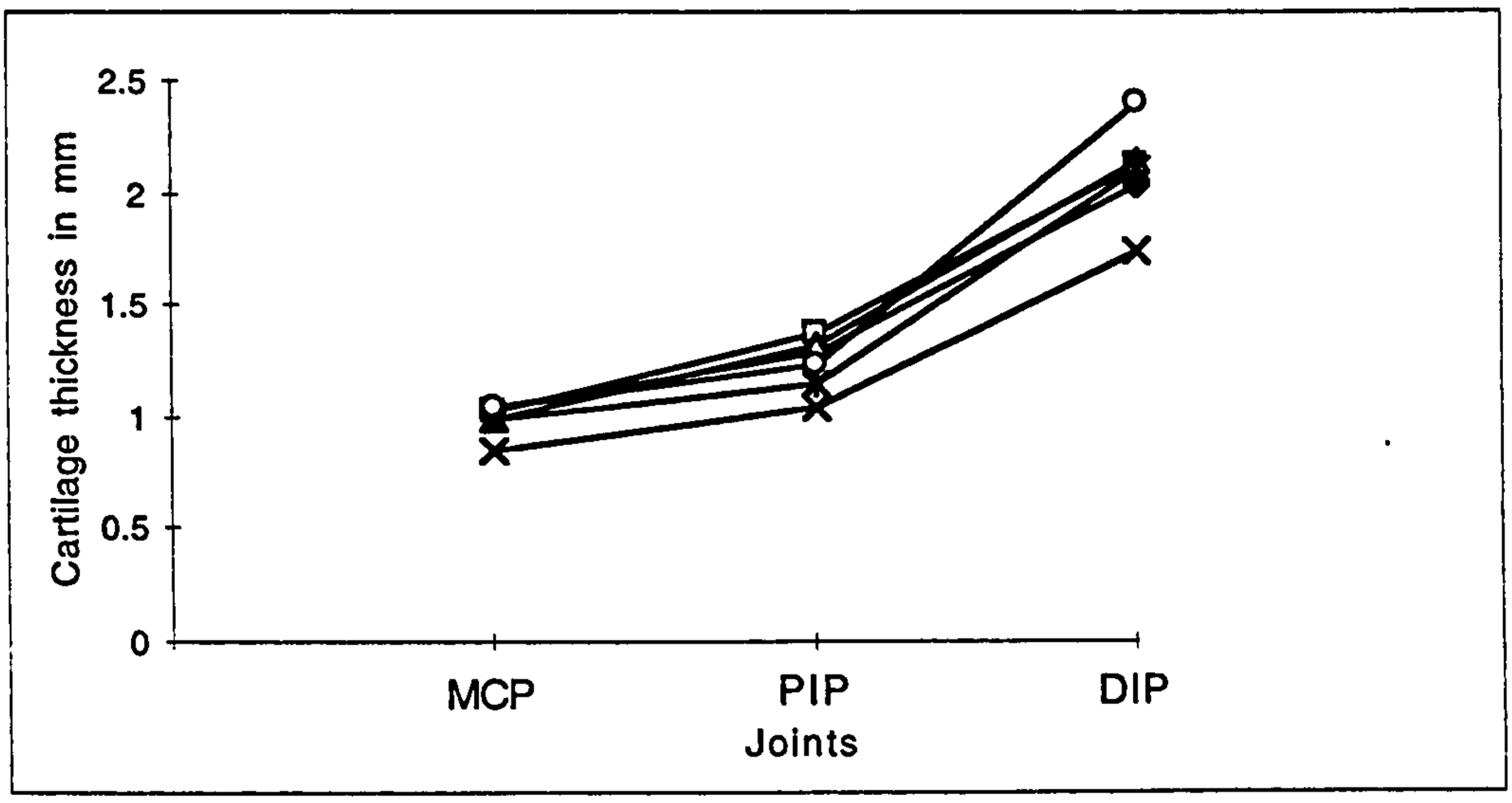

Figure 10-10: Cartilage thickness of distal surface of matched normal MCP, PIP, and DIP joints

Joint	MCP/MTP	PIP	DIP
Forelimb	3.34(0.43)	2.18(0.23)	4.46(0.32)
Hindlimb	3.09(0.67)	1.54(0.4)	4.06(0.97)

Table 10-5: Mean (SD) cartilage mass in grams of cartilage from fore and hindlimb normal joints.

For abbreviations see Table 10-4

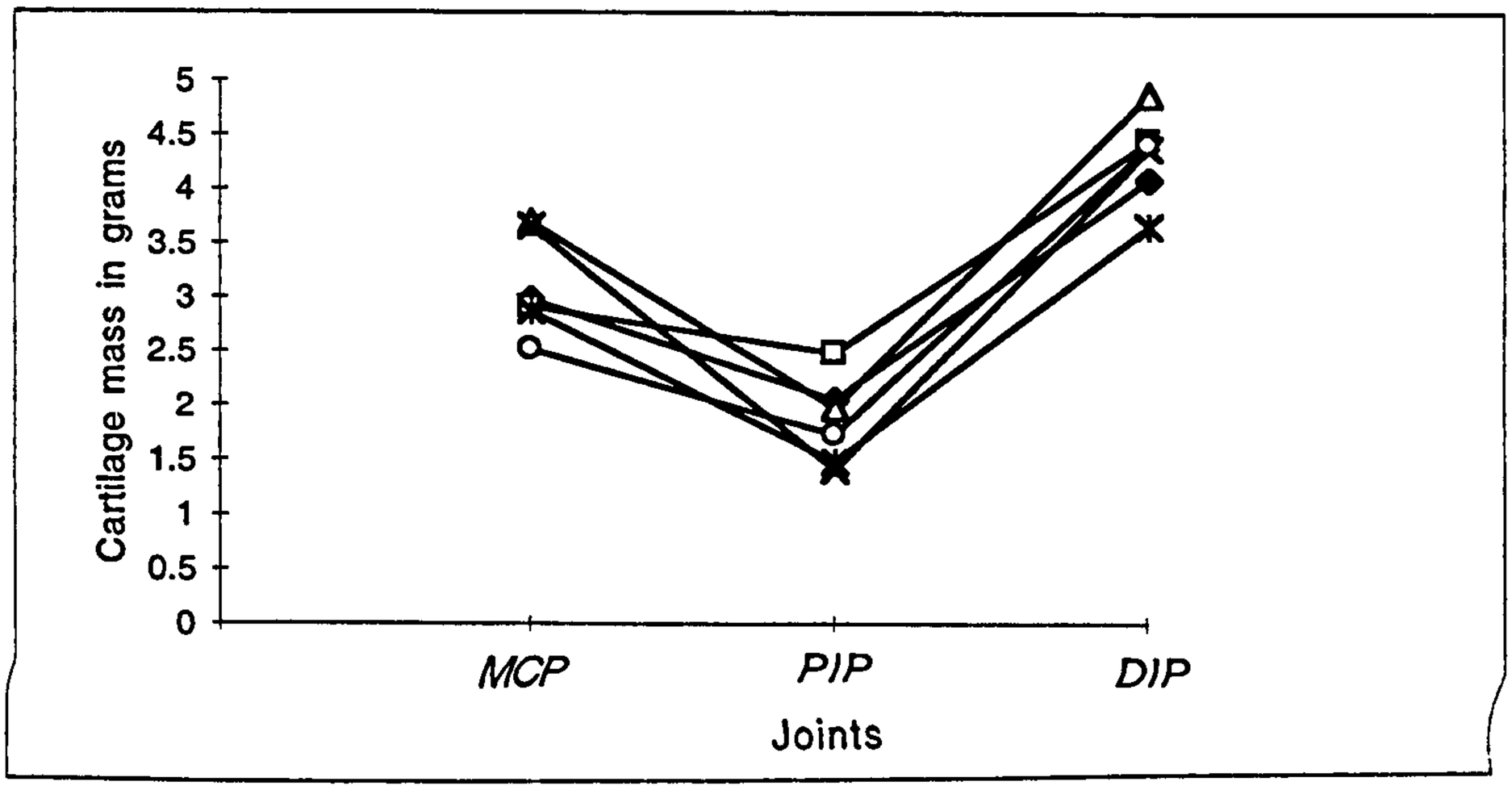

Figure 10-11: Mass of cartilage from both surfaces of matched normal MCP, PIP, and DIP joints.

Results show a significant difference ($p < 0.001$) between the thickness of cartilage in the MCP, PIP, and DIP joints. There is a similar significant difference ($p < 0.001$) between the cartilage thickness of the hindlimb MTP, PIP and DIP joints. There is no significant difference between the cartilage thickness in joints from the forelimb as compared with the equivalent hindlimb joint.

There is a significant difference, $p < 0.001$, between the cartilage mass of the MCP , PIP, and DIP of the forelimb, and $p < 0.02$ between the hindlimb MTP , PIP and DIP joints.

Discussion

Variation in metabolism

The data reported here clearly indicate a significant difference in the rate of release of GAG from the matrix of normal cartilage from these three different joints, as well as an increased rate of response of cartilage from different joints to the same challenge by interleukin -1. This implies an intrinsic difference in the cartilage from the MCP, PIP, and DIP joints, since without any stimulation from external cytokines, the turnover of GAG *in vitro* is greater in the DIP joint than the PIP, which in turn is still greater than that of the MCP. What causes this difference is not understood.

The cartilage explants used in this study were all from the same age group of horses, 5 out of the 6 horses were estimated to be 10 -15 years old. Interestingly, the cartilage from horse 4 which was 20 years old was the least responsive to the effect of IL-1. This age effect has previously (MacDonald *et al.* 1992) been reported where explants obtained from older horses were considerably less responsive to IL-1 at a similar dose rate of 10ng/ml, both in respect of synthesis and release of proteoglycan.

The extracellular matrix of cartilage is produced by the chondrocytes which also control matrix synthesis and degradation and therefore turnover. Chondrocyte metabolism is controlled by both genetic factors and environmental circumstances, such as changes in the composition of the extracellular matrix and physical stimuli. Diffusion of substances within the matrix depends on molecular size, shape and charge as well as concentration of proteoglycans in the cartilage. Proteoglycan concentration increases with depth of cartilage (Mow *et al.* 1992) probably in order to resist compressive strain. The thickness and possibly also shock absorbence of the cartilage increases from MCP, to PIP, and further to DIP in the same order as the rate of release of GAG from normal cartilage explants *in vitro* increase. It is possible, although maybe simplistic to think that a higher normal rate of GAG release is simply related to a higher concentration of proteoglycan in the cartilage. However the results of this study show that the rate of release of GAG also varies between these joints in response to addition of IL-1 to the explant system.

It could be that the altered environmental conditions caused by removal of cartilage from the *in vivo* to the *in vitro* state may cause upregulation of the cytokines mediating normal turnover of proteoglycan. However, all the explants in this study were prepared in the same

way and exposed to the same conditions, so these factors do not provide an explanation for the differences reported here. Alterations in the matrix composition e.g. a higher concentration of proteoglycan could affect the diffusion rate of IL-1, although at 17kDa it is a relatively small molecule.

The normal turnover of proteoglycan depends on a balance between proteinases and proteinase inhibitors. This normal equilibrium is thought to be upset in the pathogenesis of OA. It is well reported elsewhere (Pelletier *et al.* 1993) that IL-1 causes an increase in cartilage degradation and a decrease in proteoglycan synthesis by the upregulation of metalloproteinases. The effects of IL-1 on chondrocytes are mediated by a single high affinity receptor (Lotz *et al.* 1995). It is possible that the numbers or sensitivity of IL-1 receptors may differ between joints. Chondrocytes also produce the IL-1 receptor antagonist, which competitively inhibits IL-1 binding to its receptor and this too may be produced in different amounts by different joints. It has recently been shown that chondrocytes from human knee and ankle joints differ in response to IL-1 and IL-1 receptor antagonist (Hauselmann *et al.* 1993), and this seems to be the most likely explanation for the differences reported in this study. The response of cartilage to IL-1 may also be mediated by the presence of other extracellular regulators that can enhance or inhibit its effect (Lotz *et al.* 1995).

This work has not demonstrated a difference in MMP-2 release from cartilage obtained from different joints of the distal limb of the horse, no matter whether stimulated with IL-1α or not. It would appear unlikely that, in this model of cartilage degradation, MMP-2 is affecting all of the aggrecan breakdown and thus GAG release. Further work is now required using monoclonal antibodies to neoepitopes of aggrecan to identify at which site aggrecan is being cleaved to help identify which types of enzymes are causing the release of GAGs in the horse (Fosang *et al.* 1996). Another approach which could be used to identify the enzyme which is involved in the GAG release from cartilage of different joints is the addition of specific MMP inhibitors to the culture media (Kozaci *et al.* 1997).

An interesting finding from this work is the variation in response of cartilage from different horses to the effect of IL-1α stimulation. Cartilage obtained from horse 2 always demonstrated an extremely high MMP-2 release with IL-1α stimulation, which was always statistically significant, no matter from which joint this was obtained. In addition, in horse 2 samples, a lot of active MMP-2 was also produced from stimulated cartilage explants, in addition to MMP-2 zymogen (results not shown). Cartilage from the PIP joint from horse number 4 and from the DIP joint in horse number 5 also demonstrated statistically significant

increase in MMP-2 production upon IL-1α stimulation in comparison to the unstimulated cartilage. Cartilage from all the other horses and sites tested demonstrated no statistically significant alteration in MMP-2 production with IL-1α stimulation. The reason for the difference in response with different cartilage samples to IL-1α stimulation is unclear, especially why all the samples from horse 2 appeared to have such a large response. We can hypothesise that the response to IL-1α stimulation requires having both IL-1 receptors available on the cells, and a relative lack of IL-1 receptor antagonists (Pelletier *et al.* 1993). It is of interest that the cartilage from horse 2 should demonstrate similar responses, no matter from what site it was obtained, tending to indicate that the causal factors to the reaction in this case are systemic rather than local to an individual joint.

Interestingly, GAG release from the explants obtained from horse 2 demonstrated an extremely high response to IL-1α stimulation (Table 10-2). Thus it would appear that the explants obtained from horse 2 were particularly sensitive to IL-1α stimulation, and this stimulation did cause a large up-regulation of MMP-2 (zymogen and active) production as well. It is impossible from this data to form a link between the increase in enzyme production, and the release of GAGs from the explants.

Whatever the reason for the alteration in response between these three joints , it clearly indicates that there are differences in cartilage metabolism. This may be the result of adaptation to different environmental influences existing within these joints, but the increased sensitivity to the action of IL-1α also suggests a possible reduction in resistance of cartilage to the degradative influence of this cytokine, and maybe therefore an increased susceptibility to disease. Further studies need to be carried out into the rate of synthesis of GAG in the MCP, PIP, and DIP joints in order to assess the rate of GAG turnover more thoroughly, as well as investigations into the number and sensitivity of IL-1 receptors existing in these joints. An epidemiological survey to establish the prevalence of osteoarthritis in these three joints in the horse would also be valuable.

Structural variation

The results from the study on normal levels of keratan sulphate in SF (Chapter 7) show an increase in ratio of this marker to total glycosaminoglycans from MCP to PIP and further to the DIP joint. It has been reported that there is an increased concentration of keratan sulphate in the deeper levels of thicker cartilage. The increase in cartilage thickness from MCP to PIP and then to DIP joint reported here may therefore be relevant to the ratio changes.

The mass of cartilage is least in the PIP joint, and increases in the MCP and further in the DIP joint. This finding therefore does not support one view that the difference in markers reported in Chapter 7 is simply related to a difference in mass of cartilage between these joints.

Despite the fact that further work must be done to investigate the differences in joint metabolism and structure reported here and the reasons for this variation , it is apparent from this study that joints are not biologically the same, and this must be taken into account when designing clinical trials and assessing therapies.

Summary

1. An explant system was set up for 7 days to investigate the hypothesis that cartilage from different equine joints may respond differently to challenge with interleukin-1 (IL-1α). The % GAG released and the response to challenge from the cartilage of the PIP and DIP joints was significantly higher ($p < 0.02$ ANOVA) than that of the MCP joint. These findings indicate that cartilage from the MCP joint may be less reactive than that from the PIP or DIP joints to the same IL-1 challenge. No significant differences were found in the activity of MMP-2 between the three joints.

2. Structural differences in cartilage thickness and mass do exist between the equine MCP, PIP, and DIP joints. Changes in cartilage thickness may explain the differences in marker ratios reported in Chapter 7 but there is no direct relationship between the markers measured and the cartilage mass.

3. Differences between joints must be taken into account when designing clinical trials.

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Chapter Eleven

General conclusions and future studies

Were the aims achieved?

Aims:

1. To carry out a controlled, blinded, pilot clinical trial in order to assess the validity and reliability of the present commonly used measures of outcome in the horse, i.e. functional outcome and radiography, and the more recent scintigraphic and molecular marker techniques, as methods of measuring changes in OA occurring in horses in clinical trials, and to evaluate a potential disease modifying drug in osteoarthritis .

A prospective, controlled, randomised and double blinded pilot study ,with clear inclusion criteria, was conducted. This allowed for the assessment of the validity and reliability of the chosen measures of outcome, and for the identification of some areas of difficulty .

a) Functional outcome

The scoring of lameness was found to be a valid and reliable measurement tool, both intra- and inter-observer. This subjective measurement, is the most common method of assessing the degree of limb pain in the horse and is used regularly in clinical trials and yet assessment of its validity has not previously been reported in the equine literature. Having established that lameness scoring is a valid method of measuring functional outcome it would be interesting to compare this method with an objective form of gait analysis, e.g. force plate studies.

The use of owner questionnaires, employing Likert scales, was also found to be a reliable and valid method of measurement of change within the trial. Physician and owner global scores of outcome correlated well. No previous reports of owner assessment of disability in equine osteoarthritis (OA) could be found in the literature. It would be beneficial to develop these questionnaires to provide more information on outcome in future clinical trials.

b) Imaging

Scoring of joint space narrowing, osteophytes, and global scoring of osteoarthritic change on radiographs was found to be reproducible cross-sectionally but was not useful in identifying change during this trial. Despite this, the radiography scoring system would seem to be the present method of choice for measurement of structural outcome.

Qualitative scoring of scintigraphic scans was only reliable intra-observer. Although scintigraphy was useful as an additional diagnostic technique in some cases, it was not helpful in measuring change during the trial and could not replace radiography. The use of the quantitative method of scintimetry in assessing the scintigraphy results was reliable but did not offer any essential additional information.

The lack of significant correlations between imaging measurements, i.e. scintigraphy or radiography, and the clinical changes measured by global scores of lameness improvement, has been reported many times in previous studies and was not unexpected.

More study is necessary in the interpretation of scintigraphic bone scans and the effect of variables such as limb positioning and blood flow. Improvement in interpretation could be made by a study of normal scintigraphic differences in the joints of clinically sound horses. With improvement in the available software packages for the interpretation of scans, adjustments for variables could be made more easily.

Calcium pentosan polysulphate (CaPPS) was not found to be an effective disease modifying drug in osteoarthritis (DMOAD) in this study. Having carried out this pilot study it will now be acceptable to assess the drug further by continuing the trial over a longer time period, both in order to recruit a larger number of cases to provide a sample size to give the trial adequate power and also to allow for more meaningful changes in the imaging measures of outcome. It should be noted that the majority of cases recruited were in stages of chronic OA which was less than ideal, since it did not allow for the comparison of the effectiveness of measurement techniques in the early diagnosis of OA.

c) Molecular markers in serum and synovial fluid

Molecular markers were not found to be useful in the measurement of change in joint status during the trial period, however, the trial did allow for the discovery of some interesting cross-sectional differences and correlations with other measurement parameters (see point 3).

Overall, in the trial carried out in this study, lameness scoring was considered to be the most useful measure of change. Radiography, scintigraphy, and molecular markers were useful in diagnosis of OA but not in measuring change over time.

2. To establish normal parameters of molecular markers in equine serum and in synovial fluid from different joints.

A range of normal values of molecular markers in serum and in different equine joints has been established. There does not appear to be a diurnal rhythm of serum hyaluronan (HAS) in the horse. However, the wide variation in normal values of HAS that does exist both within and between horses, and the effect of variables such as age and exercise on this marker make it unlikely to be useful as a serum marker of OA in the horse.

We have found that there is a change in the ratio of keratan sulphate (KS-5D4) to total glycosaminoglycans (GAG) in the SF of normal equine joints, increasing from the metacarpophalangeal (MCP), to the proximal interphalangeal (PIP), and further to the distal interphalangeal (DIP) joints. The normal concentrations of hyaluronan (HA) and cartilage oligomeric matrix protein (COMP) within the MCP, PIP, and DIP joints also varies. While it is vital that these normal differences are taken into account when interpreting marker changes in OA, it is possible that these variations relate to a difference in metabolism between the cartilage in these joints, a finding which highlights the potential use of markers as tools to measure the natural history of OA.

3. To investigate the use of molecular marker technology to assess changes in joint status and disease process both cross-sectionally and in longitudinal change in OA.

Markers measured in this study were found to be valuable in identifying cross-sectional differences between normal and OA joints. Measurement of HA, KS, GAG , KS:GAG ratio, and bone specific alkaline phosphatase (BAP) in synovial fluid were all significantly different in clinically active OA joints when compared to contralaterals. Concentrations of BAP, KS, and the KS:GAG ratio also demonstrated significant correlations with articular cartilage pathology, an important finding which adds validity to their use as markers of joint pathology and in the case of BAP, demonstrates a link between subchondral bone and articular cartilage

disease status. This is the first study to report the use of synovial fluid BAP measurements in the horse.

Differences in concentrations of these markers in contralateral control joints also identified differences between populations of horses, which may have been related to training, breed, or age.

HA concentration in synovial fluid was found to correlate with radiography and scintigraphy scores, but temporal changes in markers were not useful measurements of OA change in the trial, and did not correlate with changes in any other parameter. However, concentration of COMP in serum at the beginning of the trial was found to correlate with the global score of lameness improvement and could therefore be said to be predictive of OA change.

4. To investigate whether there is variation in the metabolism of different normal equine joints, by the examination of response to challenge by the articular cartilage .

In vitro cartilage studies demonstrated that there is a significant difference in the rate of release of GAG from the matrix of normal cartilage from the MCP, PIP, and DIP joints, as well as a difference in susceptibility of cartilage from these different joints to the same challenge by interleukin -1. This implies an *intrinsic* difference in the cartilage from the MCP, PIP, and DIP joints. These findings may provide evidence that cartilage physiology is specific to the function required by the particular joint. No difference was found between the three joints in the activity of the gelatinase MMP-2.

To establish whether there are differences in GAG synthesis as well as in degradation, cartilage from these three joints could be used in *in vitro* culture systems to examine the relative synthesis of GAG by ^{35} sulphate labelling *in vitro*. This would increase the understanding of potential differences in the overall turnover of GAG.

A further project relating to the investigation of metabolic joint differences could involve the assessment of the relative prevalence and risk factors of osteoarthritis in the MCP, PIP, and DIP joints both in a population of riding horses and in racehorses. This could be conducted by questionnaire, using the Likert scale, and would include our definition of osteoarthritis,

and detailed questions to establish the methods used in accurate diagnosis of the disease, the joints affected and in which type of horse.

Summary

This study has served to highlight the correct methodology for clinical trials and has identified some of the problem areas when dealing with equine cases. Functional outcome measured by lameness scoring, and radiography scoring cross-sectionally, have been proved to be reliable, but improvements need to be made in interpretation of scintigraphic data. Biochemical markers were not effective in the longitudinal assessment of osteoarthritic change, but were very useful in gaining insight into joint status and identifying cross-sectional differences. Work done on establishing normal ranges of markers, the correlation between markers and articular cartilage pathology, and the changes in marker levels occurring in the OA joint has formed a basis for the validation of these markers as measures of outcome in clinical trials.
The study has provided evidence that joints are biologically different, by both the investigation of synovial fluid marker levels, and the response of the articular cartilage to challenge.

There is a requirement in equine research for basic epidemiological studies into the incidence and prevalence of equine OA and for well planned clinical trials using previously validated measures of outcome. This study has established that these trials should be joint specific, has validated the use of lameness scoring, and confirmed that radiography scoring is reliable. It is hoped that this information will assist in improving the standard of equine clinical trials, which may lead to progress in the search for a more effective therapy for OA in the horse.

"In research the horizon recedes as we advance, and is no nearer at sixty than it was at twenty. As the power of endurance weakens with age, the urgency of the pursuit grows more intense.....And research is always incomplete."

Mark Pattison 1813 - 1884